WESTERN SYDNEY UNIVERSITY

Understanding vancomycin tolerance in hospital-acquired methicillin-resistant *Staphylococcus aureus* isolates from patients with sustained bacteraemia

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IV. List of Abbreviations:

Abbreviation	Full term
ºC	degrees Celsius
%	percent
<	less than
≤	less than or equal to
>	greater than
≥	greater than or equal to
agr	accessory gene regulator
ARMEG	Antimicrobial Resistance Mobile Elements Group
ASM	American Society for Microbiology
ATCC	American Type Culture Collection
AUC	area under the curve
β	beta
BHIA	brain heart infusion agar
BHIB	brain heart infusion broth
Ca^{2+}	calcium ion
CAMHB	cation-adjusted Mueller-Hinton broth
CFU	colony forming units
CLSI	Clinical and Laboratory Standards Institute
e.g.	*exempli gratia*; 'for example'
et al.	*et alia;* 'and others'
g	gram(s)
h	hour(s)

HBA	horse blood agar
hVISA	heterogenous vancomycin-intermediate *Staphylococcus aureus*
i.e.	*id est*; 'that is'
L	litre
LBA	Luria-Bertani agar
µg	microgram(s)
µL	microliter(s)
MBC	minimum bactericidal concentration
MDK	minimum duration for killing
mg	milligram(s)
Mg^{2+}	magnesium ion
MHA	Mueller-Hinton agar
MHAV	Mueller-Hinton agar containing 2 µg/mL vancomycin
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
min	minute(s)
mL	millilitre(s)
MRSA	methicillin-resistant *Staphylococcus aureus*
PAP	population analysis profiling
PBP2a	penicillin-binding protein 2a
PVL	Panton-Valentine leukocidin
RPAH	Royal Prince Alfred Hospital
rpm	revolutions per minute
s	second(s)
ST	sequence type

TSB	tryptic soy broth
VISA	vancomycin-intermediate *Staphylococcus aureus*
VSSA	vancomycin-susceptible *Staphylococcus aureus*

V. List of Definitions

Term	Definition
Isolates 220-post and 225-post	Two tolerant strains that underwent an *in vitro* vancomycin passage step in order to revive the tolerance phenotype. Identified as Sa0793 and Sa0794 respectively. Parent strains are isolates 220 and 225.
Isolates 220 and 225	Two reported tolerant strains supplied from the United States that were non-tolerant upon repeat testing from storage. Identified as Sa0795 and Sa0796 respectively.
Sustained bacteraemia	Interchangeable with persistent and/or prolonged bacteraemia which are terms used in the literature to describe bacteraemia lasting 3 to 7 days from onset. All isolates used in this study are from patients with sustained bacteraemia episodes lasting 5 or more days from onset
Isolate type: Initial	Isolate obtained at onset from a sustained bacteraemia episode
Isolate type: Persistent	Recurring isolate(s) obtained during the course of a sustained bacteraemia episode
Isolate type: Recurrent	Isolate obtained after completed treatment of sustained bacteraemia episode; following discontinuation of therapy
Tolerance (MBC)	Bacteria that test susceptible but evade the killing of an antibiotic; often represented as a MBC:MIC of $\geq$32. This study classified strains with a MBC:MIC ratio $\geq$16 as tolerant if the MIC was 8 µg/mL (due to assay vancomycin concentration endpoint of 128 µg/mL).
Tolerance (Time-kill) CLSI definition	Following antibiotic exposure: Greater than a 3 log reduction in bacterial population at 24 h
Tolerance (Time-kill) Brauner *et al.* definition	Following antibiotic exposure: Greater than MDK_{90} of 6 h.

VI. Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of healthcare-associated infections including bacteraemia. Vancomycin has traditionally been the antibiotic of choice for treatment, but despite using the minimum inhibitory concentration (MIC) to guide treatment management, some patients still fail antibiotic therapy even when infected with strains that test as susceptible. This is further exacerbated with the emergence of vancomycin intermediate *S. aureus* (VISA) and heterogeneous vancomycin intermediate *S. aureus* (hVISA) strains, which exhibit reduced susceptibility to vancomycin. When exposed to sub-lethal concentrations of vancomycin, these VISA and hVISA subtypes are believed to undergo cell wall metabolism changes which increases the thickness of their cell walls and this form of resistance has been associated with sustained bacteraemia and increased mortality.

Antibiotic tolerance is a phenotypic trait where an organism is resistant to the lethal killing of an antibiotic despite being inhibited by normal concentrations, and may explain the poorer outcome seen in patients with sustained bacteraemia. *In vitro* this interaction between an antibiotic concentration and bacterial growth or killing can be measured by the MIC (antibiotic concentration required to inhibit bacterial growth) and the minimum bactericidal concentration (MBC; that antibiotic concentration required to kill the bacterium) respectively. Using these two measures, tolerance is defined when the ratio of an isolates measured MBC to MIC is $\geq$32 after 24 hrs of incubation.

Despite guidelines for MBC testing being issued in 1999, there is ongoing variation in the methodology used among testing laboratories, and the disparity in reported tolerance rates among *S. aureus* strains has lead clinicians to question the utility of MBC testing.

Although there is conflicting data on the clinical significance of vancomycin-tolerant staphylococci, evidence suggests that tolerance may be an independent risk factor for poorer outcome, and the close association with hVISA and VISA strains may provide an understanding of the mechanisms that drive vancomycin resistance.

This study investigated whether variations in the methodology used in MBC and time-kill tests impacted the ability to detect antibiotic tolerance. This was achieved determining the effect of using different media and test conditions on tolerance rates seen in a collection of hVISA and VISA isolates from an ST239 dominant MRSA population.

The results from this study demonstrate that MBC results vary between tests and that the detection of antibiotic tolerance is highly dependent on testing conditions. Time-kill assays are the recommended method for detecting tolerance, and if MBC testing were to be performed, then it should be performed after isolates have undergone an *in vitro* vancomycin pre-exposure step.

To better understand antibiotic tolerance and the mechanisms that drive vancomycin reduced susceptibility, MBC testing must be better standardised, and furthermore, the findings from this thesis propose an enhancement to current testing methodology which can be used in future large-scale studies to determine the clinical relevance of antibiotic tolerance.

VII. Statement of Authenticity

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Signed 

Date: 08 / 03 / 2017

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IX. Publications arising from this thesis

1. Dimitrijovski B, Jensen SO, Espedido BA, van Hal SJ. 2015. “Tolerance” of misused terminology? Enforcing standardised phenotypic definitions. mBio 6(3):e00446-15. doi:10.1128/mBio.00446-15.

X. Publications to arise from this thesis (to be submitted)

1. Dimitrijovski B, Jensen SO, Espedido BA, Gosbell, IB, van Hal SJ. 2016. *Staphylococcus aureus* tolerance testing: In search of the optimal method. To be submitted to J Antimicrobial Chem.

XI. Presentations arising from this thesis

1. Australian Society for Microbiology Clinical Special Interest Group, Sydney, October 2013. Dimitrijovski B. Vancomycin tolerance in *Staphylococcus aureus*: Fact or fiction?

2. Department of Microbiology, Royal Prince Alfred Hospital, Camperdown, June 2016. Dimitrijovski B. Understanding vancomycin tolerance in hospital-acquired *Staphylococcus aureus* isolates from patients with sustained bacteraemia.

Chapter 1

Introduction

1.1 Background

S. aureus is a gram-positive bacterium which is found as part of the normal microorganism flora of human skin, and is generally non-pathogenic in healthy individuals. However, *S. aureus* is an opportunistic pathogen which can infect both immunocompromised and immuno-competent people, and has the ability to produce a variety of toxins and virulence factors in addition to having the ability to become resistant to antimicrobial agents (Chambers and Deleo 2009).

S. aureus is recognised as a pathogen of great concern, as strains are often associated with various virulence factors and multi-drug resistance (Reis, Eisencraft *et al.* 1995, Kullar, Davis *et al.* 2011). *S. aureus* readily evolves resistance by utilising a number of mechanisms against multiple antibiotic types, and excessive antibiotic use has led to a high number of multi-resistant strains (Harris, Foster *et al.* 2002). For example, penicillin was introduced in the early 1940s for treating *S. aureus* infections, however resistance to penicillin resulting from the penicillinase enzyme encoded by the *blaZ* gene was seen as early as 1942 (Lowy 2003). Methicillin was introduced in the United Kingdom in 1957 as an alternative for treating penicillin-resistant *S. aureus* infections, and resistance was seen within a few years of use. These methicillin-resistant *S. aureus* (MRSA) strains harbour the *mecA* gene, which is responsible for the synthesis of penicillin-binding-protein 2a (PBP2a). These proteins have a low affinity to β-lactam antibiotics, and these MRSA strains can still synthesize their cell wall even in the presence of methicillin (Hiramatsu 1995). Vancomycin, a glycopeptide antibiotic often used as a last resort for treatment, is now widely used to treat bacteraemia caused by MRSA strains, and as a consequence, reduced susceptibility and resistance to vancomycin have been reported (Hiramatsu, Hanaki *et al.* 1997, Chang, Sievert *et al.* 2003).

1.2 *S. aureus* bacteraemia

1.2.1 Risk factors

S. aureus is a major cause of bacteraemia; in Australia, 6,900 episodes of *S. aureus* bacteraemia occur annually, with 24% of these cases being caused by MRSA strains (Turnidge, Nimmo *et al.* 2007). These MRSA infections are strongly associated with sustained bacteraemia (bacteraemia lasting from 3 up to 7 days from onset), higher hospital costs, a longer duration of stay, and higher mortality and morbidity (Lodise and McKinnon 2005, Moise, Sakoulas *et al.* 2007, Kullar, Davis *et al.* 2011, Pasticci, Moretti *et al.* 2011, Hope, Blackburn *et al.* 2013). Sustained bacteraemia is a common manifestation of bloodstream infections caused by *S. aureus* (van Hal, Jensen *et al.* 2012), and while admission to ICU and the presence of a central venous catheter or other foreign bodies such as a pacemaker or prosthetic joint are recognized as risk factors (Hawkins, Huang *et al.* 2007, Honda, Doern *et al.* 2011), infection with hVISA/VISA isolates has been more closely associated with sustained bacteraemia (Howden, Johnson *et al.* 2006, Rybak, Leonard *et al.* 2008). Sustained bacteraemia is associated with an enhanced risk of additional complications such as infective endocarditis, and poorer outcomes and higher mortality rates have been seen in these patients compared to patients with non-sustained infections (Hawkins, Huang *et al.* 2007, Yoon, Kim *et al.* 2010).

1.2.2 Endocarditis

As a result of high bacterial loads, sustained bacteraemia substantially increases the risk of developing infective endocarditis (Holland, Arnold *et al.* 2014); loads which are similar to what is seen in hVISA bloodstream infections (Charles, Ward *et al.* 2004, Howden 2005). Cases of MRSA endocarditis are invariably bacteraemic, and poorer patient outcomes are seen in patients with MRSA endocarditis and is associated with a fatal outcome if improperly treated (Kullar, Davis *et al.* 2011, Pasticci, Moretti *et al.* 2011, Holland, Arnold *et al.* 2014).

1.2.3 Genetic markers for sustained bacteraemia

There are no genetic markers in *S. aureus* which can predict a sustained bacteraemia infection or treatment failure. Studies have suggested the accessory gene regulator (*agr*) locus of *S. aureus*, which acts as a global virulence regulator, as an important mechanism affecting vancomycin susceptibility (Sakoulas, Eliopoulos *et al.* 2003, Moise-Broder, Sakoulas *et al.* 2004, Sakoulas, Moise-Broder *et al.* 2004, Verdier, Reverdy *et al.* 2004, Rose, Fallon *et al.* 2012), but the relevance of *agr* dysfunction in cases of sustained bacteraemia is still unclear (Kullar, Davis *et al.* 2011, Seidl, Chen *et al.* 2011). Even *S. aureus* strains that possess the highly potent toxin generated from the Panton-Valentine leukocidin (PVL) gene has not been associated with sustained bacteraemia nor endocarditis (Holmes, Ganner *et al.* 2005, Kullar, Davis *et al.* 2011).

1.2.4 Relevance of vancomycin therapy

Vancomycin is a glycopeptide antimicrobial and is the most widely used agent as therapy for serious infections caused by gram-positive organisms (Jones 2006). Vancomycin derives from the organism *Streptomyces orientalis*, and acts by inhibiting the bio-synthesis of peptidoglycan (bacterial cell wall) and the assembly of N-acetylglucosamine and N-acetylmuramic acid (NAM-NAG) polypeptide into the growing peptidoglycan chain. It inhibits peptidoglycan bio-synthesis by reacting with D-Ala-D-Ala, blocking the release of terminal D-Ala and intra-bond formation, resulting in cell death (Chakraborty, Sahu *et al.* 2010). Vancomycin was initially used to treat infections with penicillin-resistant *Staphylococcus aureus* before alternative, less-toxic drugs were introduced. However with the onset of antimicrobial resistance, highlighted by increasing reports and hospital outbreaks of methicillin resistance in *S. aureus* strains in the 1960s, there was a greater dependency on vancomycin for treating these infections. While vancomycin was initially used as a last resort for treatment, it is now widely used as a first line therapy to treat MRSA bacteraemia (Rose, Fallon *et al.* 2012).

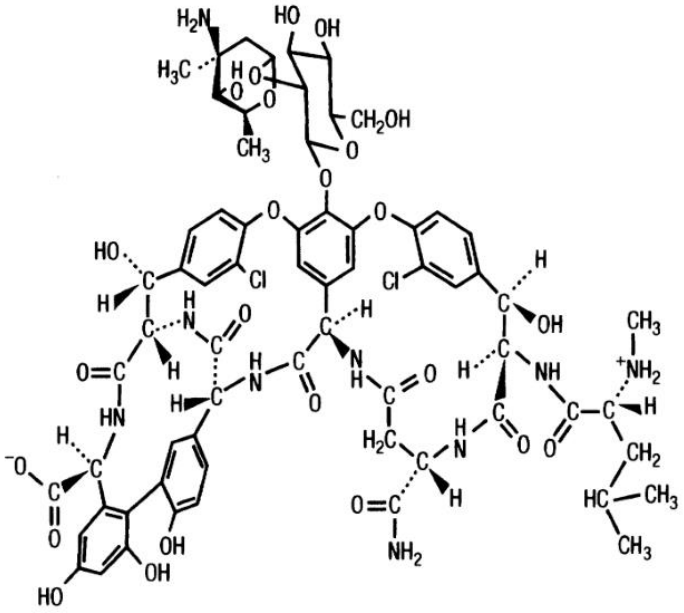


Figure 1.1 Chemical structure of vancomycin. Image sourced from Jones (Jones 2006).

Resistance to vancomycin remains rare, however there are increasing numbers of reported cases of MRSA strains which exhibit reduced susceptibility to vancomycin (van Hal, Jensen *et al.* 2012). These VISA and hVISA strains are associated with sustained bacteraemia, resulting from reduced efficacy seen with vancomycin when used to treat MRSA bacteraemia (van Hal, Jensen *et al.* 2012).

1.3 *S. aureus* strains with reduced vancomycin susceptibility

The CLSI define VISA as an *S. aureus* strain with a vancomycin MIC of 4-8 µg/mL (CLSI 2007), and hVISA are *S. aureus* strains that normally test within the vancomycin susceptible range (MIC ≤2 µg/mL), but a sub-population of organisms show intermediate resistance to vancomycin (Cosgrove, Carroll *et al.* 2004). The mechanism in which decreased susceptibility occurs in these strains is still not yet fully understood (Aeschlimann, Hershberger *et al.* 1999), though it is believed that these hVISA and VISA strains develop a thickened peptidoglycan cell wall (to which a greater number of vancomycin molecules

bind to moieties within the cell wall rather than at the active site), and consequently higher concentrations of the antibiotic are required in order to kill the bacteria (Hanaki, Labischinski *et al.* 1998). The production of these thickened cell walls is associated with increased production of cell wall precursors, decreased autolysis, increased penicillin-binding proteins and a slower growth rate (Aeschlimann, Hershberger *et al.* 1999, Joyce and Woods 2004). This contrasts the mechanism of resistance seen in vancomycin-resistant *S. aureus* (VRSA) strains, which acquire resistance by conjugal transfer of the *vanA* gene from vancomycin-resistant enterococci. Furthermore, VRSA infections are rarely associated with bloodstream infections (Gould 2010, van Hal, Jensen et al. 2012, Gould 2013).

1.3.1 Genetic correlates

The genetic resistance mechanism of VISA/hVISA isolates is still not completely understood due to the complex metabolic adaptation possessed by these strains, and the *vanA* and *vanB* genes that are responsible for vancomycin resistance in enterococci and vancomycin resistant *S. aureus* have not been associated with these isolates (Joyce and Woods 2004, Verdier, Reverdy *et al.* 2004, Jones 2006). Therefore routine clinical laboratories rely on phenotypic methods to detect these VISA/hVISA strains (van Hal and Paterson 2011).

1.3.2 Detection issues

A modified population-analysis profile is considered the gold standard for detecting VISA/hVISA strains (Wootton, Howe *et al.* 2001). However this method is labour-intensive and unachievable in a clinical laboratory setting (van Hal, Wehrhahn *et al.* 2011), and therefore laboratories rely on MIC testing recommended by the CLSI in order to detect these strains (CLSI 2012). Automated broth-based microdilution methods such as Microscan (Siemens AG, Erlangen, Germany), Phoenix (Becton-Dickinson, Franklin Lakes, USA) and Vitek (bioMerieux, Craponne, France), in addition to disc diffusion methods fail to detect VISA and hVISA strains and are no longer recommended as testing methods (Cosgrove, Carroll *et al.* 2004, Moise, Sakoulas *et al.* 2007, Rybak, Vidaillac *et al.* 2013). The CLSI recommend broth microdilution MIC testing for determining vancomycin susceptibility in *S. aureus*, however routine clinical laboratories use the Etest (bioMerieux, Craponne, France) method due to its ease of use (Lodise, Graves *et*

al. 2008, Holmes, Turnidge *et al.* 2011, Kullar, Davis *et al.* 2011, Pasticci, Moretti *et al.* 2011). While the CLSI recommend Etest as an acceptable alternative testing method, the sensitivity in detecting hVISA strains in broth microdilution and Etest methods is poor (van Hal, Wehrhahn *et al.* 2011). Laboratories have explored modifications to the Etest method such as the macromethod Etest or glycopeptide resistance detection Etest tests, though these methods still fail to reliably detect hVISA strains (van Hal, Wehrhahn *et al.* 2011). Overall, the detection issues associated with MIC testing creates additional challenges in the management of patients with *S. aureus* bloodstream infections, as infections with VISA/hVISA strains may be under reported and inappropriate therapy may be administered as a consequence (Sader, Jones *et al.* 2009).

1.4 *Staphylococcus aureus* minimum inhibitory concentration testing

1.4.1 Principle

Broth and/or agar dilution methods are used to quantitatively measure the *in vitro* activity of vancomycin against *S. aureus* (CLSI 2012). A series of tubes or plates are prepared to which various concentrations of vancomycin is added. The tubes or plates are inoculated with a standardised suspension of *S. aureus*, and after incubation at 35 $\pm$ 2ºC, the tests are examined and the MIC is determined. With the Etest method, a plastic strip containing increasing concentrations of vancomycin is placed on a Mueller-Hinton agar plate that is pre-inoculated with a suspension equivalent to a 0.5 McFarland standard. The MIC is the lowest concentration of antibiotic needed to inhibit bacterial growth, though this does not imply that the organism is necessarily killed (Finberg, Moellering *et al.* 2004, Joyce and Woods 2004, CLSI 2012). Vancomycin MIC interpretive criteria for *S. aureus* is listed in Table 1.1.

Table 1.1 CLSI guidelines for determining vancomycin susceptibility in *S. aureus* (CLSI 2007)

Organism	Antimicrobial agent	MIC interpretive criteria (µg/mL)[1]			Comments
		S	I	R	
S. aureus	Vancomycin	≤2	4-8	≥16	MIC tests should be performed to determine the susceptibility of all isolates of staphylococci to vancomycin. The disc test does not differentiate vancomycin-susceptible isolates of *S. aureus* from vancomycin-intermediate isolates. Disc diffusion testing is not reliable for testing vancomycin

[1]MIC, (minimum inhibitory concentration); S, (susceptible); I, (intermediate); R, (resistant)

1.4.2 Differences in methodology

Broth microdilution is considered the gold standard for susceptibility testing and is the method used by the CLSI when establishing vancomycin MIC breakpoints. Due to the ease of use however, Etest is the method of choice for routine clinical diagnostic laboratories when determining vancomycin MICs (Kullar, Davis *et al.* 2011, Pasticci, Moretti *et al.* 2011, Rose, Fallon *et al.* 2012); favoured over the broth microdilution method which is considered too laborious and inconvenient (Moise, Sakoulas *et al.* 2007, Kullar, Davis *et al.* 2011, Pasticci, Moretti *et al.* 2011, Rose, Fallon *et al.* 2012, Sancak 2014).

Poor correlation and a high degree of variability is seen in the Etest method (Sancak 2014), and MICs obtained via Etest are consistently higher when compared to the broth microdilution method (Sakoulas, Moise-Broder *et al.* 2004, Holmes, Turnidge *et al.* 2011, Kullar, Davis *et al.* 2011, Rose, Fallon *et al.* 2012).

1.4.3 Vancomycin susceptibility and relationship with therapy

While hVISA and VISA strains are closely associated with high mortality and sustained bacteraemia (Sakoulas, Moise-Broder *et al.* 2004, Moise, Sakoulas *et al.* 2007, Lodise, Graves *et al.* 2008, Holmes, Turnidge *et al.* 2011, Kullar, Davis *et al.* 2011), there are conflicting reports of treatment failure and increased mortality associated with vancomycin MICs near the cut-off for susceptibility (i.e. MIC of 1.5-2 µg/mL) (Moise, Sakoulas *et al.* 2007, van Hal, Jensen *et al.* 2012, Murray, Zhao *et al.* 2013). Many studies that correlate increased-but-susceptible vancomycin MICs with poorer patient outcomes base their findings on the Etest method, and studies have attributed poorer outcomes seen in MRSA bacteraemia to a phenomenon known as 'MIC creep' (Hawkins, Huang *et al.* 2007, Dhand and Sakoulas 2012), where gradual increases in vancomycin MIC have been reported over time but are still below the breakpoint for susceptibility. However, as the Etest method is known to produce higher MICs compared to the broth microdilution method and is unable to detect small increments in the MIC in the clinical setting (Hawkins, Huang *et al.* 2007, Hope, Blackburn *et al.* 2013), this phenomenon has been heavily criticized (Jones 2006, Pasticci, Moretti *et al.* 2011, Reynolds, Hope *et al.* 2012). Furthermore, studies have refuted this trend of increasing vancomycin MICs over time, citing retrospective literature searches and testing of earlier isolates, which both demonstrate long-term presence of VISA, pre-dating even the first reported case in 1997 (Hiramatsu, Hanaki *et al.* 1997, Rybak, Cha *et al.* 2005, Jones 2006).

Varied treatment outcomes have been seen in patients with sustained bacteraemia caused by MRSA strains exhibiting a high-but-susceptible vancomycin MIC (Jung, Song *et al.* 2014). It has been argued whether the current vancomycin MIC susceptible breakpoint of $\leq$2 µg/mL should be lowered further (Moise, Sakoulas *et al.* 2007), or whether there may be additional organism or host factors, not solely the vancomycin MIC affecting the clinical response.

The efficacy of vancomycin has also been questioned, as this antibiotic has been referred to as having suboptimal activity (van Hal, Jensen *et al.* 2012), and treatment failure has been reported when insufficient concentrations of vancomycin are administered during therapy (Kullar, Davis *et al.* 2011, Gonzalez, Sevillano *et al.* 2013).

In conclusion, there are no definitive correlates which can predict the treatment efficacy of vancomycin in patients infected with sustained bacteraemia caused by MRSA strains. An alternative explanation, in addition to understanding the reduced vancomycin susceptibility seen in VISA/hVISA strains, may depend on whether the organism possess an ability to evade the bactericidal killing of vancomycin; an *in vitro* phenomenon known as vancomycin tolerance (Jones 2006, Cazares-Dominguez, Cruz-Cordova et al. 2015).

1.5 Vancomycin tolerance

Vancomycin tolerance is a novel form of resistance which improves the chance of bacterial survival; a phenotypic trait where bacterial growth is being inhibited and it continues to survive despite increasing antibiotic concentrations (Sabath, Wheeler *et al.* 1977, Handwerger and Tomasz 1985). This is in contrast to resistance where both growth and bacterial survival are not affected by standard antibiotic concentrations. *In vitro*, this interaction between an antibiotic concentration and bacterial growth or killing can be measured by the MIC (antibiotic concentration required to inhibit bacterial growth) and the MBC (antibiotic concentration required to kill the bacterium), respectively. Using these two measures, tolerance is defined when the ratio of an isolates measured MBC to MIC is $\geq$32 after 24 h of incubation (May, Shannon *et al.* 1998, CLSI 1999, Sader, Jones *et al.* 2009, Honda, Doern *et al.* 2011).

1.6 MBC

1.6.1 Introduction

Vancomycin tolerance is determined by investigating bacterial survival that occurs beyond the point of an organism's MIC; referred to as an MBC test. As such, MBC testing involves the transfer of any clear MIC wells onto solid media in order to determine cell viability, and similar to broth microdilution MIC testing, has high costs associated with the test and is too labour-intensive to be performed in routine clinical laboratories (Miyazaki, Takata *et al.* 2011).

1.6.2 Factors that influence the MBC test

The methodology for MBC testing is subject to many biological and technical factors, and, as such, the clinical significance is hard to determine (Taylor, Schoenknecht *et al.* 1983, Pelletier 1984, Washington 1988). While the clinical relevance of biological factors is uncertain, technical factors are believed to contribute to the lack of correlation and varied results seen in MBC tests, raising criticisms toward the accuracy and significance of the test (Kaye 1980, Sherris 1986, May, Shannon *et al.* 1998, Pasticci, Moretti *et al.* 2011). In an attempt to address these factors and establish inter-laboratory reproducibility and reliability, the Clinical and Laboratory Standards Institute (CSLI) in 1999 issued recommendations for MBC testing (CLSI 1999) which was followed by methodology, essentially reflecting the CLSI guidelines, published by the American Society for Microbiology (ASM) (Garcia 2010).

1.6.3 Biological factors

1.6.3.1 Persister cells

Persisters occur when a small number of bacterial cells survive the lethal killing of an antibiotic, but are just as susceptible as the parent strain and no greater proportion of persistence is seen upon repeat testing (Gunnison, Fraher *et al.* 1964). This is thought to occur as a result of slower-growing cells that are not killed by the antibiotic, and as the rate of killing is related to the rate of bacterial growth, the antibiotic will

have a reduced killing effect (Tuomanen, Cozens *et al.* 1986). Therefore, as growth of a microorganism reaches its maximum, its growth rate slows and so does the rate of bactericidal killing (CLSI 1999).

1.6.3.2 Paradoxical effect

The paradoxical effect is where the proportion of survivors increase with each increasing antibiotic concentration beyond the MBC. This phenomenon is common in *S. aureus* and for cell-wall active agents, where penicillin-binding inefficiencies prevent organism growth from the full bactericidal killing of an antibiotic (French 2006). The clinical relevance of the paradoxical effect is unclear and is ignored when seen in *S. aureus* (CLSI 1999).

1.6.4 Technical factors

1.6.4.1 Growth phase of inoculum

The most common variation between MBC studies is the growth phase of bacteria used for testing. Stationary phase cultures (for example, cultures that are >8 h) will include a number of dormant cells which are not as susceptible and therefore cause diminished killing rates. Lag phase cultures and cultures that have undergone a change in test conditions such as change in temperature or inoculation from solid to liquid media, will also include less metabolically active cells, resulting in less killing endpoints. Several studies strongly recommend logarithmic phase growth for MBC testing, and the CLSI state this is achieved for staphylococci within 6 h (Goessens, Fontijne *et al.* 1982, Taylor, Schoenknecht *et al.* 1983, Sherris 1986, CLSI 1999).

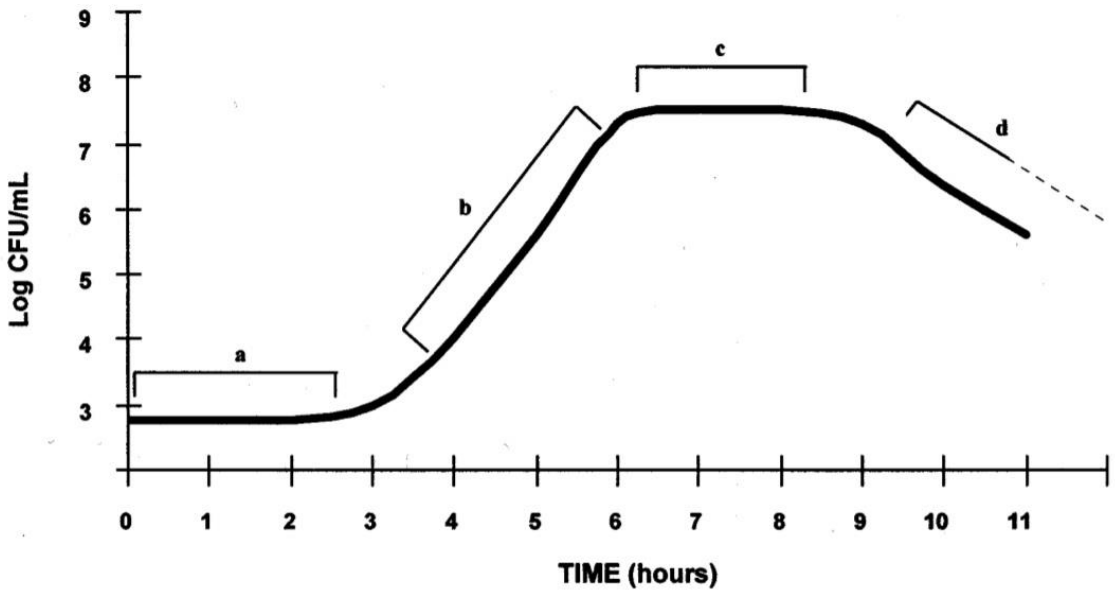


Figure 1.2 Bacterial growth curve. Growth curve representing a typical bacterial culture. a: lag phase; b: logarithmic phase; c: stationary phase; d: death phase. Image sourced from CLSI (CLSI 1999).

1.6.4.2 Size of inoculum

Inoculum size is one of the most important variables that can affect MBC testing. Low concentrations of bacteria (e.g. 10^4 CFU/mL) are readily killed, and high concentrations of log-phase cultures (10^7-10^8 CFU/mL) are killed more rapidly than those in stationary phase (CLSI 1999). Variations in inoculum density can also affect MIC endpoints, and therefore the CLSI recommend a final inoculum size of 5 x 10^5 CFU/mL (CLSI 2012).

Inocula from log phase culture should be prepared in a flask or beaker which is continuously agitated in a shaker incubator to ensure uniform growth. While the inoculum size can be estimated on the day of testing against a known McFarland reference standard, the final inoculum size of approximately 5 x 10^5 CFU/mL should be confirmed by colony count for interpretation of killing endpoints the following day (CLSI 1999).

1.6.4.3 Insufficient contact

During testing, organisms may adhere to the microtitre well (or test tube) above the meniscus, leading to insufficient contact between the organism and antibiotic. Mixing (or vortexing) at 20 h or continuous agitation in a shaker incubator allows for better contact between the organism and antibiotic (CLSI 1999).

1.6.4.4 Antibiotic carryover

MBC testing requires the transfer of clear MIC wells onto solid media, and while bacteria is being transferred during this step, so is antibiotic. Antibiotic carryover can occur at higher concentrations (>16X MIC) and can be detected by inoculating the test broth across the surface of a dried agar plate (allowing 20 minutes for absorption), then cross-streaking the inoculum over the entire surface of the plate. After 24 h of incubation, inhibition of colony growth would be investigated at the site of the initial streak.

1.6.4.5 Volume transferred

The volume used to transfer from clear MIC wells should be such that at least 10 colonies are counted after 99.9% killing; for example, a 99.9% killing endpoint for a starting inoculum of 5×10^5 CFU/mL would be 100 colonies (CLSI 1999).

Antibiotic carryover can generate false negative results, particularly if volumes $\geq$0.1 mL are being transferred, and volumes $\leq$0.01 mL can result in too few colonies being transferred. Though the CLSI recommend a transfer volume which is between 0.01-0.1 mL, MBC rejection values and the sensitivity and specificity of colony counts (which factor pipetting error and Poisson distribution of sample responses) are calculated at a transfer volume of 0.01 mL (Pearson, Steigbigel *et al.* 1980, CLSI 1999).

1.6.4.6 Choice of media

Artificial media has little in common with the interstitial fluid of the patient, and the constitution of media used can influence the result due to variations in pH, proteins, osmolality, salt concentrations, and divalent cations. Therefore, cation-adjusted Mueller-Hinton broth (CAMHB) is recommend for MBC testing as it

resembles serum in pH, osmolality and cations, compared to other broth media (CLSI 1999, CLSI 2012). The pH should be monitored prior to MBC testing to ensure it is between 7.2 and 7.4.

1.6.4.7 Effect of storage

Prolonged storage at temperatures ≤-70ºC can affect an organism's ability to retain the tolerance phenotype. In some cases the effect of tolerance can diminish, and in others tolerance can be lost entirely (Mayhall and Apollo 1980). When taken from storage, the CLSI recommend sub-culturing an isolate three times prior to MBC testing (CLSI 1999).

1.6.5 Method

As the broth microdilution method is considered the gold standard for the determination of MICs, therefore, MBC testing is also performed using this method. The microdilution method is simple, more efficient, reduces the amount of broth needed, and unlike the macrodilution method, adherence of organisms to the sides of wells does not appear to be a problem (Taylor, Schoenknecht *et al.* 1983, Ampel, Keating *et al.* 1984). Furthermore, greater reproducibility and reliability is seen in the microdilution method, despite being more difficult to determine the 99.9% killing endpoint (Shanholtzer, Peterson *et al.* 1984).

MBCs are determined by first performing the standard broth dilution technique for MICs. After 24 h incubation any clear wells (i.e. wells demonstrating inhibited growth), are inoculated onto a blood agar plate and the number of colonies are recorded. The MBC is determined by a 99.9% reduction of colonies compared to the starting concentration (CLSI 1999, Joyce and Woods 2004, Honda, Doern *et al.* 2011, Rose, Fallon *et al.* 2012), and a bactericidal drug is expected to achieve this within 2 dilutions of the organism's MIC (Joyce and Woods 2004).

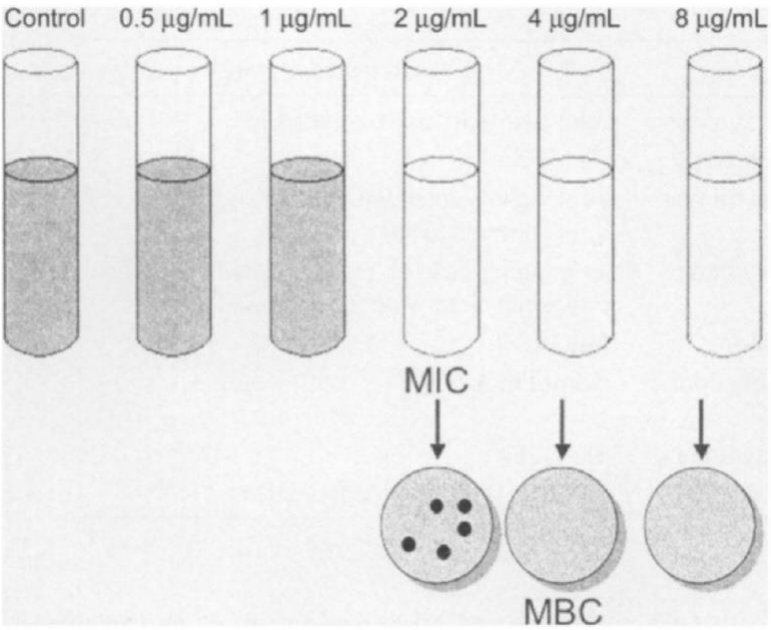


Figure 1.3 MIC and MBC – Macrodilution method. The MIC is defined by the first clear tube (or well) of an antibiotic dilution series, whereas the MBC is defined by the antibiotic concentration in which >99.9% bacteria is killed. This figure represents an MIC of 2 µg/mL and MBC of 4 µg/mL, MBC:MIC = 2. Image sourced from Finberg *et al.* (Finberg, Moellering *et al.* 2004)

1.6.6 Issues with MBC testing

Despite the attempts to standardise MBC testing (CLSI 1999, Garcia 2010), guidelines still allow for variability. For example, to achieve a logarithmic-phase culture the CLSI guidelines recommend *S. aureus* strains are pre-incubated for 'up to six hours' prior to testing, despite data showing increased paradoxical effect and increased number of survivors when strains are incubated greater than 5 h (Taylor, Schoenknecht *et al.* 1983). Additionally better reproducibility has been demonstrated when the pre-incubation step is set at 3 h (May, Shannon *et al.* 1998). Therefore, unlike MIC testing that has been widely adopted and accepted in diagnostic laboratories, MBC testing is viewed with scepticism partly due to the unreliability of methods used and lack of confidence in the results obtained (May, Shannon *et al.* 1998).

There are also variations on the definition of antibiotic tolerance. Although vancomycin tolerance is generally accepted as vancomycin MBC to MIC ratio of ≥32 after 24 hours of incubation (Voorn, Kuyvenhoven *et al.* 1994, May, Shannon *et al.* 1998, CLSI 1999, Sader, Jones *et al.* 2009, Honda, Doern *et al.* 2011), there have been variations of this definition which include a MBC:MIC ratio of ≥16 (Rajashekaraiah, Rice *et al.* 1980, Honda, Doern *et al.* 2011), ≥8 (Gonzalez, Sevillano *et al.* 2013), or a MBC value alone of ≥16 µg/mL (Sabath, Wheeler *et al.* 1977).

Reported rates of vancomycin tolerance among clinical *S. aureus* strains vary between laboratories and range from 4-50% (Reis, Eisencraft et al. 1995, Honda, Doern et al. 2011, Pasticci, Moretti et al. 2011, Rose, Fallon et al. 2012, Gonzalez, Sevillano et al. 2013). One study alone showed a variation in the prevalence of vancomycin tolerant strains from 20.1% to 24.8%, depending on the numerical definition being used (Sader, Jones et al. 2009). Therefore, the variability of MBC test procedures in addition to the various definitions used could explain the differences in the proportion of reported tolerant strains.

Aside from the technical and definition issues, the main criticism of MBC testing is that a 99.9% kill at 24 hours is an arbitrary endpoint and does not show the rate of kill (Kaye 1980). Time-kill assays instead show the true killing response which fits a sigmoidal pattern with a trend for a gradual reduction in the number of surviving CFU over time (Liu, Zhang *et al.* 2004). Time-kill assays also show that the one dimensional endpoint associated with MBC testing represents the least accurate part of a kill curve, and that the MBC test fails to detect strains that are killed more slowly than others (Sherris 1986). Therefore, time-kill assays are considered a more reliable means for determining tolerance in *S. aureus* (Handwerger and Tomasz 1985).

1.7 Time-kill

1.7.1 Principle

The time-kill assay is a method that can assess the bactericidal activity of an antibiotic, and can establish the rate at which an antibiotic kills the isolate (Garcia 2010). Time-kill assays can be used to confirm the paradoxical effect, distinguish between tolerance and persistence (Figure 1.4), and can be used to explain treatment failures and predict clinical outcome (Brauner, Fridman *et al.* 2016). However, the disadvantage of time-kill assays is that the concentration of antibiotic is fixed over the killing period, which can differ from the concentration achieved within a patient during the administering of the antibiotic (Firsov, Zinner *et al.* 2007).

Compared to the MBC test, there are fewer technical deficiencies and a higher degree of reproducibility in a time-kill assay, and time-kills tend to be preferred when determining the bactericidal efficacy of vancomycin (May, Shannon *et al.* 1998, CLSI 1999, Moise, Sakoulas *et al.* 2007).

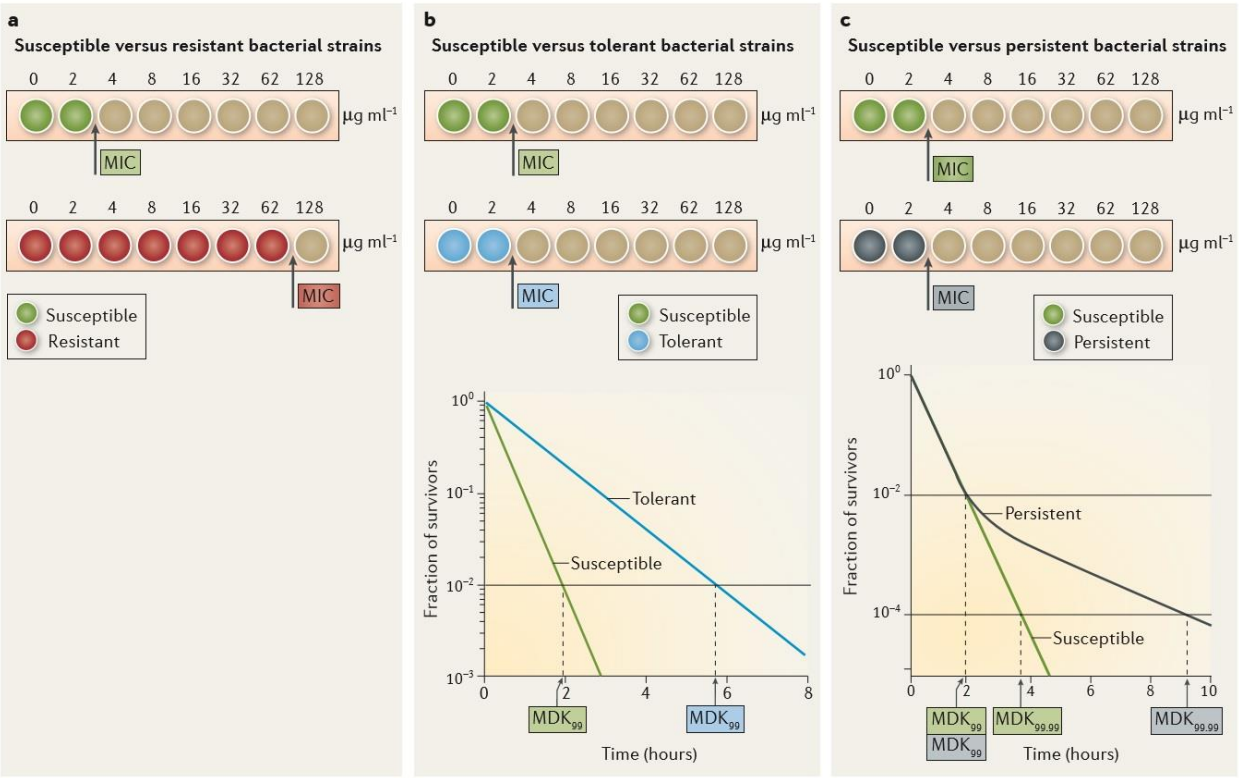


Figure 1.4 Differentiation between resistance, tolerance and persistence. a) The MIC for a resistant strain is much higher than a susceptible strain. In this example the MIC of the resistant strain is 128 µg/mL compared to the susceptible strain which is 4 µg/mL. b) The MIC for a tolerant strain is similar to that of a susceptible strain (4 µg/mL), however the minimum duration for killing (MDK) of 99% of colonies is higher for a tolerant compared to a non-tolerant strain. c) The MIC for a persistent strain is similar to that of a susceptible strain (4 µg/mL), however the MDK for 99.9% of colonies is higher for the persistent strain when compared to the susceptible strain. Image sourced from Brauner *et al.* (Brauner, Fridman *et al.* 2016).

1.7.2 Method

Time-kill assays are typically performed in glass tubes containing 10 mL of CAMHB with a fixed concentration of antibiotic, and another tube without antibiotic which is used as a growth control (CLSI 1999). The inoculum is prepared similar to that of a MBC test (i.e. logarithmic-phase culture) and must be

added in a manner that avoids splashing within the tube. Larger vessels such as flasks, beakers and bottles allow for a larger volume of broth to be tested and result in a greater challenge to the antimicrobial agent and therefore are not recommended (CLSI 1999).

Whilst the CLSI recommend testing antibiotic concentrations at multiple intervals of the MIC, studies tend to favour using a vancomycin concentration of 16 µg/mL, particularly because it reflects the concentration that can be achieved in serum (May, Shannon *et al.* 1998, Aeschlimann, Hershberger *et al.* 1999, Joyce and Woods 2004, Sakoulas, Moise-Broder *et al.* 2004, Moise, Sakoulas *et al.* 2007)

Sampling for colony counts is achieved by removing 0.5 mL samples from the broth at specified times, and the CLSI recommend sampling time intervals at 0, 4, 8, 10-12, and 24 h of incubation. Tubes should be vortexed before sampling in order to re-suspend bacteria adhering to the wall. Samples are serially diluted in 4.5 mL of sterile 0.9% NaCl to produce a dilution series (e.g. 10^{-1} to 10^{-4}), and no more than 0.1 mL from each serial dilution is removed and plated onto a blood agar plate, streaked, and cross-streaked 20 minutes later, which determines the CFU/mL (CLSI 1999). Prolonged incubation of the blood agar plate (e.g. 48 h) facilitates better colony reading of smaller colonies; some of which the hVISA population are an example of (CLSI 1999).

Recorded colony counts can be charted on semi-log paper, with the survivor colony count on the ordinate in logarithmic scale and the time on the abscissa in arithmetic scale (CLSI 1999), but are better represented by charting the fraction of survivors over the elapsed time, in hours (Figure 1.5) (May, Shannon *et al.* 1998, Brauner, Fridman *et al.* 2016).

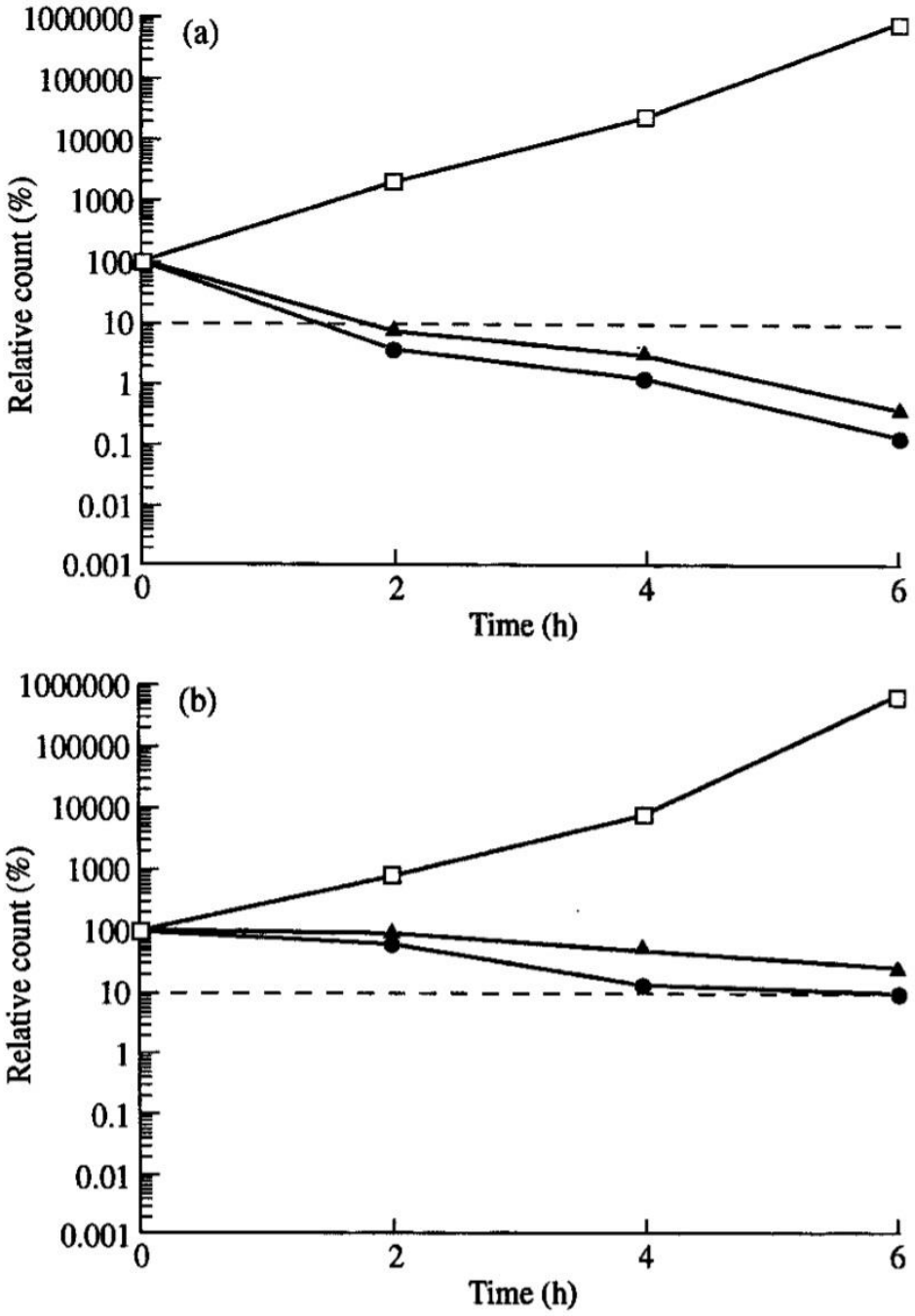


Figure 1.5 Time kill curves. Killing of *S. aureus* isolates a) non-tolerant strain 112 and b) tolerant strain 119 by glycopeptides. The kill rate is monitored over times 0, 2, and 6 hours. Symbols: □, growth control; ▲, vancomycin (20 µg/mL); ●, teicoplanin (10 µg/mL). The fraction of survivors is represented by the percentage relative count. Image sourced from May *et al.* (May, Shannon *et al.* 1998).

1.7.3 Issues with time-kill assays

1.7.3.1 Antibiotic carry-over

Antibiotic carry-over can be a problem with higher antibiotic concentrations ($\geq$4X MIC) and is detected by observing for inhibition of growth at the site of inoculation (Figure 1.6). Serial dilutions in saline and drying the inoculum on the agar surface before streaking both can reduce the effect of antibiotic carry-over, or alternatively bacterial cells can be washed prior to plating. (CLSI 1999).

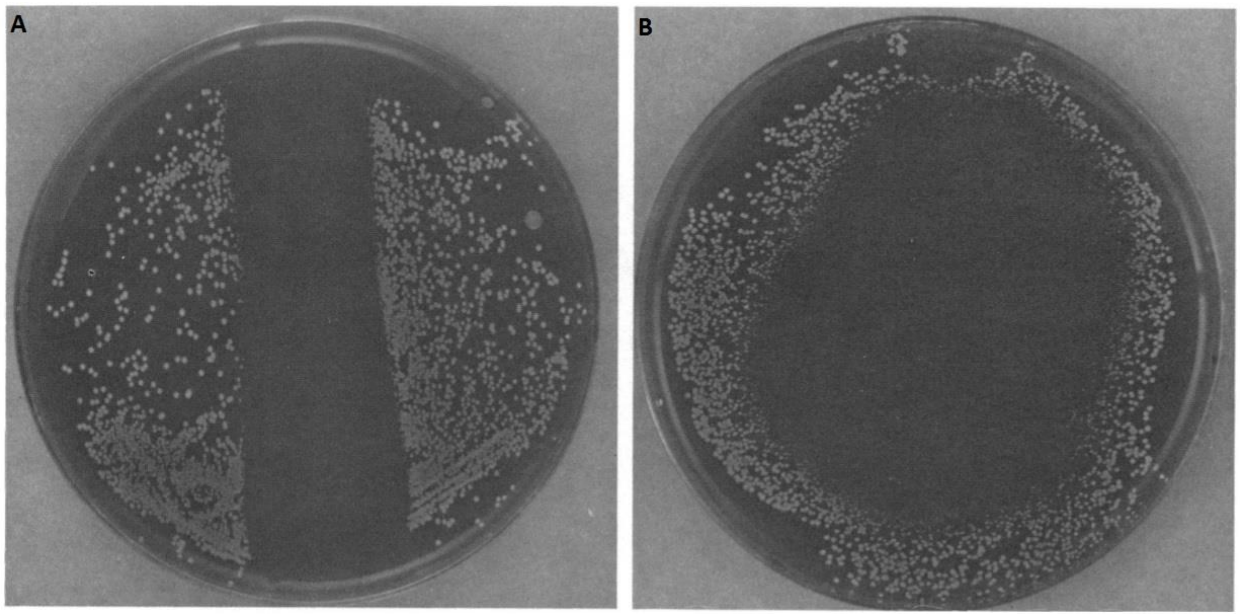


Figure 1.6 Antibiotic carry-over effect. Inoculum containing antibiotic was spread in a blood agar plate. a) Allowing the streak to be absorbed into the agar prior to spreading. b) Spread immediately without being absorbed. The antibiotic carry-over effect is more noticeable when the inoculum is spread immediately. Image sourced from Shanholtzer *et al.* (Shanholtzer, Peterson *et al.* 1984).

1.7.3.2 Survivors

Over time, colony counts may increase after an initial decrease which can be due to the selection of resistant mutants, inactivation of the antimicrobial agent, or regrowth of susceptible bacterial cells which have escaped antimicrobial activity by adhering to glass tube (CLSI 1999). The clinical importance of this regrowth is unclear, particularly if it occurs after the usual administered dosing interval of the antibiotic. The significance may depend on the time at which regrowth occurs, the dosing of the antibiotic in the clinical setting, the type of bacteria, and the antibiotic itself.

Determining the MIC of survivors will demonstrate if a selection of resistant mutants has occurred, whereas assaying the antibiotic potency in the broth at times 0 and 24 h will detect whether the antibiotic has become inactivated (CLSI 1999). To detect antibiotic inactivation, MICs can be performed from the 0 and 24 h broth with an ATCC control strain and if the MIC increases in the 24 h sample, then the antibiotic has been deactivated.

1.7.3.3 Interpretation of time-kills

The interpretation of time-kills is difficult, and comparing results of an isolate with another isolate from a similar clinical case is the most predictive way to evaluate results (CLSI 1999). Furthermore, there are differences in the criteria used to define tolerance using time-kill data. The CLSI for example defines tolerance as a < 3-$\log_{10}$ reduction in bacterial count at 24 h (CLSI 1999). May and colleagues however, have suggested based on linear regression models a corresponding assessment point (i.e. <2-log reduction at 6 h) better represents the true killing rate, prevents false positives secondary to bacterial regrowth of isolates which can occur between 6 and 24 h, and prevents false negatives due to loss of nutrients and decreased growth (May, Shannon *et al.* 1998).

1.8 Antibiotic tolerance

1.8.1 Clinical relevance

The *in vivo* clearance rate of bacteraemia caused by *S. aureus* strains appears to be related to the rate of bactericidal killing achieved by vancomycin *in vitro* (Moise, Sakoulas et al. 2007). Antibiotic tolerance results in a lower bactericidal kill and lower percentage of bactericidal activity which adversely affects the outcome of serious *S. aureus* infections treated with vancomycin. In some cases the risk of mortality is increased (Sorrell, Packham et al. 1982, Reis, Eisencraft et al. 1995, Aguilar, Gimenez et al. 2009), and in other cases additional antibiotics were necessary for cure (Gopal, Bisno *et al.* 1976, Faville, Zaske *et al.* 1978). Inversely, increased bactericidal killing has been associated with rapid clearance of bacteraemia and lower all-cause mortality (Sakoulas, Moise-Broder et al. 2004). Tolerance is also significantly more common in patients with endocarditis which has been associated with therapeutic failure and increased mortality (Denny, Peterson *et al.* 1979, Kaye 1980, Rajashekaraiah, Rice *et al.* 1980, May, Shannon *et al.* 1998, Pasticci, Moretti *et al.* 2011), even despite the use of additional antibiotics during therapy (Wilson and Gaya 1996).

1.8.2 Link between tolerance and reduced susceptibility

Vancomycin tolerance is more common in MRSA than MSSA strains (May, Shannon *et al.* 1998). It is believed that the PBP2a associated with MRSA strains alters cell wall metabolism, slowing the rate of growth, and generates a thickened cell wall. This change in cell wall metabolism is also theorised as the mechanism for reduced vancomycin susceptibility in VISA and hVISA strains, and could explain the association between vancomycin tolerance and reduced vancomycin susceptibility (Jones 2006, Cazares-Dominguez, Cruz-Cordova et al. 2015).

1.9 Conclusion

There are no clinical or biological markers which can predict the treatment outcome for patients with MRSA bacteraemia, and the mechanism for reduced vancomycin susceptibility is still not completely understood. Antibiotic tolerance is a phenotypic trait that allows an organism to survive lethal concentrations of vancomycin, and may explain the poor clinical response seen in these patients.

The ongoing uncertainty about the best methodology has lead clinicians to question the utility of MBC testing, and antibiotic tolerance assessment being relegated to "curiosity" status as a consequence. However, several studies have observed significantly poorer patient outcomes in infections, especially with MRSA isolates, that show vancomycin tolerance (Denny, Peterson et al. 1979, Lodise, Drusano et al. 2014, Britt, Patel et al. 2017). With antimicrobial resistance increasing globally and the declining development of new and novel antibiotics, a greater emphasis on better usage of current antimicrobials has arisen. As tolerance reflects one component of the spectrum from susceptibility to total resistance, MBC and tolerance testing might provide vital information that can inform clinicians and lead to better antibiotics, particularly in tolerant *S. aureus* strains which show susceptibility to vancomycin (Rose, Fallon *et al.* 2012).

1.10 Scope of Research

The aim of the study was to investigate the methodology used for tolerance testing with the aim of establishing a uniform approach for testing. This study was conducted by investigating the prevalence of vancomycin tolerance in an ST239 dominant MRSA population. Isolates were obtained from an organism storage library maintained by the Antimicrobial Resistance Mobile Elements Group (ARMEG). This culture collection consists of *S. aureus* isolates collected from patients with sustained bacteraemia, and contains a high proportion of VISA/hVISA strains previously confirmed by population-analysis profiling-area under the curve (PAP-AUC). A series of isolates collected in real-time from patients with sustained *S.*

aureus bloodstream infections were also investigated. Known tolerant strains supplied from the United States (US) (Rose, Fallon et al. 2012) were used as control organisms for MBC testing and time-kill assays.

The use of different media and test conditions were examined to determine the effect this has on tolerance detection. Antibiotic tolerance was determined by MBC testing and time-kill assays.

Chapter 2

Materials and Methods

2.1 Isolates

Bacterial strains used in this study are listed in Tables 2.1-2.4.

Retrospective isolates: 67 clinical bloodstream *S. aureus* strains were isolated from a series of sustained bacteraemia episodes (lasting ≥5 days from onset) seen in patients between 1998 and 2012 at Liverpool Hospital, Australia. Phenotypes were previously confirmed by modified population analysis profiling (PAP). Three ATCC quality control strains were used as MIC and MBC quality control indicators: American Type Culture Collections (ATCC) 29213 which is a vancomycin-susceptible *S. aureus* (VSSA), ATCC 700698, also referred to as Mu3, which is a hVISA, and ATCC 700699, also referred to as Mu50, which is a VISA. All of these strains were stored at -80°C.

Two reported tolerant strains provided by the University of Wisconsin-Madison (Rose, Fallon *et al.* 2012) were used as tolerant positive controls, however the submitting laboratory noted tolerance was lost in both strains upon culture from storage. Therefore two separate cultures per strain was received: One set was received on Mueller-Hinton agar (MHA) after direct subculture from -80°C storage; isolates Sa0795 (220) and Sa0796 (225). Another set, after subculture from -80°C storage, had tolerance induced *in vitro* by serial passage in increasing levels of vancomycin and were then plated onto 2 µg/mL vancomycin-containing Mueller-Hinton agar (MHA). These isolates were Sa0793 (220-post) and Sa0794 (225-post). Isolates Sa0793 and Sa0795 are from the same parent strain "220", and Sa0794 and Sa0796 are from the same parent strain "225".

Prospective isolates: Bloodstream *S. aureus* strains isolated from patients with sustained bacteraemia (lasting ≥5 days from onset) between 2013 and 2014 in Royal Prince Alfred Hospital (RPAH), Camperdown Australia. Isolates were collected in real time and were tested prior to being stored at -80ºC.

Isolates taken from -80ºC storage were subcultured three times on horse blood agar (HBA) (Edwards Group, Narellan NSW, AU) prior to testing, as recommended by CLSI guidelines. All tests were performed in duplicate unless indicated, and in the setting of multiple tests, all results indicate the average result.

Table 2.1 *S. aureus* isolates – Clinical strains isolated from patients in Liverpool Hospital.

Isolate Number	Isolate Type	Phenotype[1]	Isolate Number	Isolate Type	Phenotype[1]
Sa0012	Recurrent	hVISA	Sa0116	Recurrent	VSSA
Sa0016	Initial	hVISA	Sa0129	Initial	VSSA
Sa0018	Recurrent	hVISA	Sa0131	Recurrent	VSSA
Sa0020	Persistent	hVISA	Sa0134	Initial	VSSA
Sa0021	Persistent	hVISA	Sa0138	Initial	VSSA
Sa0022	Persistent	VSSA	Sa0158	Initial	VSSA
Sa0037	Initial	hVISA	Sa0160	Persistent	VSSA
Sa0040	Initial	hVISA	Sa0162	Persistent	VSSA
Sa0044	Recurrent	hVISA	Sa0164	Persistent	VSSA
Sa0047	Persistent	VSSA	Sa0192	Persistent	VSSA
Sa0048	Initial	VSSA	Sa0202	Persistent	VSSA
Sa0049	Persistent	VSSA	Sa0209	Initial	VSSA
Sa0050	Persistent	hVISA	Sa0210	Persistent	VSSA
Sa0051	Initial	hVISA	Sa0211	Persistent	VSSA

Sa0052	Persistent	hVISA	Sa0212	Initial	VSSA
Sa0053	Initial	VSSA	Sa0214	Recurrent	VSSA
Sa0055	Initial	VSSA	Sa0228	Initial	VSSA
Sa0056	Recurrent	hVISA	Sa0265	Persistent	VSSA
Sa0057	Initial	VSSA	Sa0283	Initial	VSSA
Sa0058	Persistent	hVISA	Sa0294	Initial	VSSA
Sa0059	Persistent	VISA	Sa0308	Recurrent	VSSA
Sa0060	Persistent	hVISA	Sa0322	Initial	VSSA
Sa0061	Initial	hVISA	Sa0323	Initial	VSSA
Sa0062	Persistent	hVISA	Sa0324	Persistent	VSSA
Sa0063	Initial	hVISA	Sa0325	Persistent	VSSA
Sa0064	Initial	hVISA	Sa0329	Initial	VSSA
Sa0065	Initial	hVISA	Sa0330	Recurrent	VSSA
Sa0066	Initial	VISA	Sa0331	Initial	VSSA
Sa0067	Recurrent	hVISA	Sa0332	Persistent	VSSA
Sa0068	Initial	hVISA	Sa0363	Initial	VSSA
Sa0069	Recurrent	VSSA	Sa0375	Persistent	VISA
Sa0070	Recurrent	hVISA	Sa0378	Persistent	VISA
Sa0091	Initial	VSSA	Sa0484	Unknown	VSSA
Sa0112	Recurrent	VSSA			

[1]Abbreviations: VSSA, (vancomycin-susceptible *S. aureus*); VISA, (vancomycin-intermediate *S. aureus*); hVISA, (heterogenous vancomycin-intermediate *S. aureus*)

Table 2.2 *S. aureus* isolates – Quality control strains.

Organism Name	Isolate Number	Phenotype[1]	Source
S. aureus	ATCC 29213	VSSA	American Type Culture Collections
Mu3	ATCC 700698	hVISA	American Type Culture Collections
Mu50	ATCC 700699	VISA	American Type Culture Collections

[1]Abbreviations: VSSA, (vancomycin-susceptible *S. aureus*); VISA, (vancomycin-intermediate *S. aureus*); hVISA, (heterogenous vancomycin-intermediate *S. aureus*)

Table 2.3 *S. aureus* isolates – Reported tolerant strains.

Organism Name	Isolate Number	Source	Comments[1]
220-post	Sa0793	University of Wisconsin-Madison	Pre-exposed to sub-lethal concentrations of vancomycin; cultured on MHA containing 2 µg/mL vancomycin
220	Sa0795	University of Wisconsin-Madison	Cultured on MHA
225-post	Sa0794	University of Wisconsin-Madison	Pre-exposed to sub-lethal concentrations of vancomycin; cultured on MHA containing 2 µg/mL vancomycin
225	Sa0796	University of Wisconsin-Madison	Cultured on MHA

[1]Abbreviations: MHA, (Mueller-Hinton agar)

Table 2.4 *S. aureus* isolates – Clinical strains isolated from patients in RPAH.

Series	Isolate Number	Isolate Type
A	**Sa0744**	Initial
A	**Sa0745**	Persistent
A	**Sa0746**	Persistent
A	**Sa0747**	Persistent
A	**Sa0748**	Persistent
A	**Sa0749**	Persistent
A	**Sa0750**	Persistent
A	**Sa0751**	Persistent
B	**Sa0752**	Initial
B	**Sa0753**	Persistent
B	**Sa0754**	Persistent
B	**Sa0755**	Persistent
B	**Sa0756**	Persistent
B	**Sa0757**	Persistent
C	**Sa0758**	Initial
C	**Sa0759**	Persistent
C	**Sa0760**	Persistent
C	**Sa0761**	Persistent
C	**Sa0762**	Persistent
C	**Sa0763**	Persistent
C	**Sa0764**	Persistent
C	**Sa0765**	Persistent
C	**Sa0766**	Persistent

2.2 Antibiotic, reagents and media

Vancomycin was purchased from a commercial source (Sigma-Aldrich., St. Louis, MO, USA). Brain-Heart infusion broth (BHIB) and Brain-Heart infusion agar (BHIA) (Oxoid Limited, Basingstoke, Hampshire, UK) were prepared as per manufacturer's instructions. Mueller-Hinton broth (MHB) (Oxoid Limited, Basingstoke, Hampshire, UK) was monitored with a pH meter prior to testing and supplemented with 20-25 mg/L calcium and 10-12.5 mg/L magnesium to ensure the pH was between 7.2-7.4. MHB with a pH reading outside this range was not used. Commercially prepared HBA and in-house prepared Luria broth agar (LBA) were used as solid plate media. Compositions for media used are listed in Table 2.5. Reagents and solutions used as well as their compositions are listed in Table 2.6.

Table 2.5 Bacteriological media.

Media	Composition	Reference
Brain-Heart Infusion agar (BHIA)	1.25% Brain infusion solids 0.5% Beef heart infusion solids 1% Proteose peptone 0.2% Glucose 0.5% Sodium choride 0.25% Disodium phosphate 1% Agar	Oxoid Limited (Hampshire, UK)
Brain-Heart Infusion broth (BHIB)	1.25% Brain infusion solids 0.5% Beef heart infusion solids 1% Proteose peptone 0.2% Glucose 0.5% Sodium choride 0.25% Disodium phosphate	Oxoid Limited (Hampshire, UK)

Luria-Bertani agar (LBA)	1 % NaCl 1 % Tryptone 0.5 % Yeast Extract 1.2% Agar	Willets and Finnegan 1970
Luria-Bertani broth (LBB)	1 % NaCl 1 % Tryptone 0.5 % Yeast Extract	Willets and Finnegan 1970
Mueller-Hinton broth (MHB)	3% Beef Extract 17.5% Casein Hydrolysate 1.5% Starch	Oxoid Limited (Hampshire, UK)
Trypticase Soy broth (TSB)	0.25% Glucose 0.17% Peptone 0.3% Peptone, soy 0.25% di-Potassium bis orthophosphate 0.5% NaCl	Becton Dickinson (New Jersey, US)

Table 2.6 Buffers and reagents.

Buffer/Reagent	Composition
1 M Calcium Chloride	1.672 g / 20 mL
1 M Magnesium Chloride	0.736 g / 20 mL
McFarland reference standard	0.5
Saline	0.90% NaCl
Vancomycin stock concentration	2,560 µg / mL

Vancomycin stock concentration was determined as described in the CLSI Methods for Dilution Antimicrobial Susceptibility Tests, M07-A9 (CLSI 2012):

$$\textit{Weight}\ (\text{mg}) = \frac{\textit{Volume}\ (\text{mL}) \times \textit{Concentration}\ (\mu\text{g/mL})}{\textit{Potency}\ (\mu\text{g/mL})}$$

As per manufacturer's certificate of analysis, the potency of vancomycin power was 996 µg/mL.

$$\text{Weight (mg)} = \frac{10\ \text{mL} \times 2{,}560\ \mu\text{g/mL}}{996}$$

Therefore 25.7 mg vancomycin power was added to 10 mL of MilliQ water to achieve a final vancomycin stock concentration of 2,560 µg / mL. This stock solution was aliquoted and stored for no more than 6 months at -20ºC. This vancomycin solution was further diluted to required concentrations and any unused aliquots were discarded after use.

2.3 Population-analysis profiling

Modified PAP-AUC as described by Wooten *et al* (Wootton, Howe *et al.* 2001) was used to determine the phenotypes for the three ATCC quality controls and the reported vancomycin tolerant control strains listed in Table 2.3. Cultures were incubated for 24 h at 37ºC in trypticase soy broth (TSB). After incubation, cultures were diluted to 10^{-3} and 10^{-6} in 0.9% sterile saline and plated on vancomycin-containing BHIA containing 0.5, 1, 2, 2.5, 4, 8, and 16 µg/mL vancomycin. After 48 h incubation at 37ºC, colonies were counted and plotted against the respective vancomycin concentration using Microsoft Excel (Microsoft Corporation; Redmond, Washington, USA). The area under the curve (AUC) was calculated for each isolate and the AUC ratio was determined by dividing an organism's AUC by the AUC for Mu3. An AUC:Mu3 ratio of $\geq$0.9 to $\leq$1.3 was used as the criteria for determining a hVISA phenotype, and a ratio of >1.3 was used as the criteria for a VISA (Howden, Davies *et al.* 2010).

A purity plate subculture, antibiotic control and negative control were used for all PAP-AUC tests. Results were deemed invalid if there was growth in the antibiotic or negative control, or if the purity plate contained mixed growth.

2.4 Time-kill studies

Time-kill studies were performed as per CLSI guidelines. 5-30 colonies obtained from an overnight HBA culture were suspended in pre-warmed MHB, shaken at 160 rpm and incubated for 3 h at 37°C to ensure logarithmic-phase growth. The turbidity was adjusted with MHB to 0.5 McFarland standard, which was then further diluted in 0.9% saline to achieve a 1-9 x 10^5 cfu/mL concentration in 10 mL MHB with and without vancomycin. Isolates inoculated in MHB without vancomycin acted as a growth controls. Cultures were shaken at 160 rpm at 37°C and incubated for 24 h. At times 0, 4, 8, 10, and 24 h and with appropriate dilutions in saline (0.5 mL from each suspension diluted in 4.5 mL saline), 100 µl from each diluted suspension was plated onto an agar plate and incubated for 48 h at 37°C. Colony counts were assessed and viable counts (in cfu/mL) were then plotted against time (in h) in Microsoft Excel. The rate of kill for each isolate was subsequently determined as previously described (May, Shannon et al. 1998, Aeschlimann, Hershberger et al. 1999), using linear regression on the observed time-kill plot with forward prediction to estimate the minimum duration to kill 99% of the bacterial population (also known as the MDK_{90}) (Brauner, Fridman et al. 2016). This was assessed against the CLSI definition of tolerance, being a < 3-$\log_{10}$ reduction in bacterial count at 24 h (CLSI 1999).

A purity plate subculture, antibiotic control and negative control were used for each assay. Results were deemed invalid if there was growth in the antibiotic or negative control, or if the purity plate contained mixed growth. The growth control was monitored by colony counts of the MHB suspension at the above specified time points. Antibiotic carry-over streaks were performed at time 0 h for each isolate: 0.1mL of the inoculated vancomycin-containing CAMHB was plated on solid media. After 15 mins, allowing for the

absorption of antibiotic into the agar, the plate was cross-streaked. After 48 h of incubation at 35ºC, plates were inspected for signs of inhibition at the initial streak in order to determine antibiotic carry-over.

2.5 MIC/MBC test

Vancomycin MICs/MBCs were determined by broth microdilution as per current CLSI guidelines. 5-30 colonies obtained from an overnight HBA culture were suspended in pre-warmed MHB, and shaken at 160 rpm and incubated for 3 h at 37°C to ensure logarithmic-phase growth. The turbidity was adjusted with MHB to 0.5 McFarland standard, which was then further diluted in 0.9% saline to achieve a final concentration of 5 x 10^5 cfu/mL. 0.01 mL of this final concentration was inoculated in a microtitre tray containing MHB and serial dilutions of vancomycin ranging from 0.5-128 µg/mL (Table 2.7). After inoculation, 0.01 mL from the growth control well was serially diluted in 0.9% sterile saline and plated on to a HBA agar plate to accurately determine the number of cfu/mL. Plates were incubated at 37°C for 48 h. Microtitre trays, stacked no more than four high, were incubated for 24 h at 37°C. All trays were shaken to dislodge any organisms adhering above the meniscus of each well at 20 h. The first well with no visible growth (i.e. the MIC) for each organism was recorded at 24 h. 10 µL of this well and all other clear wells were inoculated and spread onto an agar plate and incubated for 48 h at 37°C. Viable counts were calculated to give cfu/mL and the 99.9% endpoint for bactericidal activity was determined by the starting concentration (Table 2.8). Tolerance was defined as a MBC:MIC ratio of $\geq$32 unless indicated otherwise. For Mu50, due to the high starting MIC (8 µg/mL) and the vancomycin concentration endpoint of 128 µg/mL, a MBC/MIC ratio of $\geq$16, was accepted as indicative of tolerance provided there was sufficient growth at 24 h.

A purity plate subculture, antibiotic control, growth control and negative control were used for each assay. Results were deemed invalid if there was growth in the antibiotic or negative control, or if the growth control was clear, or if the purity plate contained mixed growth.

Table 2.7 MIC/MBC Microtiter tray.

	1	2	3	4	5	6	7	8	9	10	11	12
A – Isolate 1	AC	V (128)	V/2 (64)	V/4 (32)	V/8 (16)	V/16 (8)	V/32 (4)	V/64 (2)	V/128 (1)	V/256 (0.5)	GC	NC
B – Isolate 1	AC	V	V/2	V/4	V/8	V/16	V/32	V/64	V/128	V/256	GC	NC
C – Isolate 2	AC	V	V/2	V/4	V/8	V/16	V/32	V/64	V/128	V/256	GC	NC
D – Isolate 2	AC	V	V/2	V/4	V/8	V/16	V/32	V/64	V/128	V/256	GC	NC
E – Isolate 3	AC	V	V/2	V/4	V/8	V/16	V/32	V/64	V/128	V/256	GC	NC
F – Isolate 3	AC	V	V/2	V/4	V/8	V/16	V/32	V/64	V/128	V/256	GC	NC
G – Isolate 4	AC	V	V/2	V/4	V/8	V/16	V/32	V/64	V/128	V/256	GC	NC
H – Isolate 4	AC	V	V/2	V/4	V/8	V/16	V/32	V/64	V/128	V/256	GC	NC

Abbreviations: AC, (antibiotic control); V, (128 µg/mL vancomycin); GC, (growth control); NC, (negative control)

0.01 mL of the 1-9 x 10^5 cfu/mL organism suspension was inoculated in columns 2-11.

Table 2.8 MBC rejection values based off a single 0.01mL sample[a].

Includes 5% pipette plus full sampling error for determination of final inoculum[b]

Final Inoculum (CFU/ml)	Rejection Value[c]	Sensitivity[d] (%)	Specificity[d] (%)
1 x 10^5	3	84	83
2 x 10^5	4	87	97
3 x 10^5	6	84	98
4 x 10^5	8	89	99
5 x 10^5	11	96	99
6 x 10^5	15	99	99
7 x 10^5	17	99	99
8 x 10^5	20	99	99
9 x 10^5	23	99	99
1 x 10^6	25	99	99
2 x 10^6	47	87	99
3 x 10^6	68	84	99
4 x 10^6	91	89	99
5 x 10^6	113	96	99
6 x 10^6	136	99	99
7 x 10^6	159	99	99
8 x 10^6	182	99	99
9 x 10^6	204	99	99
1 x 10^7	227	99	99

[a] When the number of colonies from a single sample was equal to or less than the rejection value, the antibiotic was declared lethal (0.999 or greater reduction in the final inoculum).

[b] Based on a single sample for the determination of the final inoculum size.

[c] Number of colonies.

[d] Sensitivity and specificity calculated for each specific final inoculum concentration and rejection value.

Table sourced from CLSI (CLSI 1999)

2.6 Modified MBC test – gradual vancomycin exposure to organism MIC

A vancomycin pre-exposure step was used as an experimental modification to the CLSI method. Prior to MIC/MBC testing isolates were passaged for 3 days at 37ºC in doubling concentrations of vancomycin every 24 h in BHI broth, starting at dilutions below the measured MIC (i.e. 1/8; 1/4; 1/2; of MIC), followed by daily passage for 3 further days at the measured MIC. All broth cultures were shaken at 160 rpm. The final suspension was plated onto BHIA containing vancomycin equivalent to the final concentration obtained in the BHIB and incubated at 37ºC for 24 h. MIC/MBC testing was performed as described in Section 2.5 by taking 5-30 colonies from this BHIA plate.

Table 2.9 Vancomycin passage algorithm.

Isolate Number	**Day 1 MIC[1]**	**Day 2 MIC[1]**	**Day 3 MIC[1]**	**Days 4-6 MIC[1]**	**Day 7 MIC[2]**
Isolate A	V/8	V/4	V/2	V	V

[1]MIC, (minimum inhibitory concentration); V, (vancomycin concentration (µg/mL), determined by previously obtained MIC results)

[2]Cultured on BHIA containing final vancomycin concentration

2.7 Modified MBC test – 0.5x vancomycin MIC

A vancomycin pre-exposure step was used as an experimental modification to the CLSI method. Isolates were exposed to a vancomycin concentration of half the organism's measured MIC for 24 h at 37ºC in BHI broth, shaken at 160 rpm. The final suspension was plated onto BHIA containing vancomycin equivalent to the final concentration obtained in the BHIB and incubated at 37ºC for 24 h. These series of isolates were also incubated in BHI without vancomycin which acted as test controls. MIC/MBC testing was performed as described in Section 2.5 by taking 5-30 colonies from the BHIA and BHIA with vancomycin plates.

2.8 Modified MBC test – pre-enrichment step

A nutrient media enrichment step was used as an experimental modification to the CLSI method. Colonies were suspended in BHIB and shaken at 160 rpm and incubated at 37ºC for 24 h. The final suspension was plated onto BHIA and incubated at 37ºC for 24 h. MIC/MBC testing was performed as described in Section 2.5 by taking 5-30 colonies from this BHIA plate.

Chapter 3

Results

Population-analysis-profiling

3.1 Introduction

Routine susceptibility testing does not reliably detect hVISA/VISA strains, and population-analysis-profile area under the curve (PAP-AUC) as described by Wootton *et al.*(Wootton, Howe *et al.* 2001) is considered the gold standard for confirming the phenotype for hVISA strains, despite not being used in routine clinical laboratories (Aeschlimann, Hershberger *et al.* 1999, Sader, Jones *et al.* 2009, Kullar, Davis *et al.* 2011). Therefore, PAP-AUC was used to determine the phenotypes for all ATCC controls used in this study and the supplied *S. aureus* tolerant strains described in Table 2.3.

3.2 Population analysis profile confirms phenotypes

Strains 29213, Sa0795 and Sa0796 were non-viable at vancomycin concentrations of 2µg/mL (Figure 3.1). Mu3 demonstrated hetero-resistance, with half of the population surviving at a vancomycin concentration of 2.5 µg/mL and there were no surviving colonies beyond 4 µg/mL vancomycin. The population profile for Mu50 showed consistently high counts until the vancomycin concentration reached 8 µg/mL. The population for Sa0793 and Sa0794 ranged between Mu3 and Mu50; the majority of the population remained until the vancomycin concentration reached 4 µg/mL. The counts for these strains dropped to 0 cfu/mL in vancomycin concentrations of 4-8 µg/mL.

PAP-AUC results for the ATCC strains confirmed their respective phenotypes: ATCC 29213, 700698 (Mu3) and 700699 (Mu50) had a PAP-AUC of 0.43, 1, and 2.52 and were confirmed as VSSA, hVISA and VISA strains respectively.

Different PAP-AUC results were seen in the supplied tolerant strains, and the PAP-AUC varied depending on whether the isolates were passaged in vancomycin prior to PAP testing (Table 3.1). Sa0795 and Sa0796 (strains 220 and 225 which were cultivated on MHA directly from -80ºC storage) had AUC:Mu3 ratios that were <0.9 (0.46; both isolates) and were classified as VSSA strains. Sa0793 and Sa0794 (strains 220-post and 225-post, which underwent vancomycin passage and were cultivated on vancomycin-containing MHA, were both classified as VISA strains, albeit Sa0794 was close to the VISA cut-off of 1.3 (2.22, 1.31 AUC:Mu3 ratios respectively).

Table 3.1 Area under the curve.

Isolate Number	Isolate Name	AUC	AUC:Mu3	PAP-AUC Identification[1]
ATCC 29213	*S. aureus*	7.90	0.43	VSSA
ATCC 700698	Mu3	18.50	1	hVISA
ATCC 700699	Mu50	46.52	2.52	VISA
Sa0795	220	8.60	0.46	VSSA
Sa0793	220-post	41.05	2.22	VISA
Sa0796	225	8.48	0.46	VSSA
Sa0794	225-post	24.21	1.31	VISA

[1]Abbreviations: VSSA, (vancomycin-susceptible *S. aureus*); VISA, (vancomycin-intermediate *S. aureus*); hVISA, (heterogenous vancomycin-intermediate *S. aureus*)

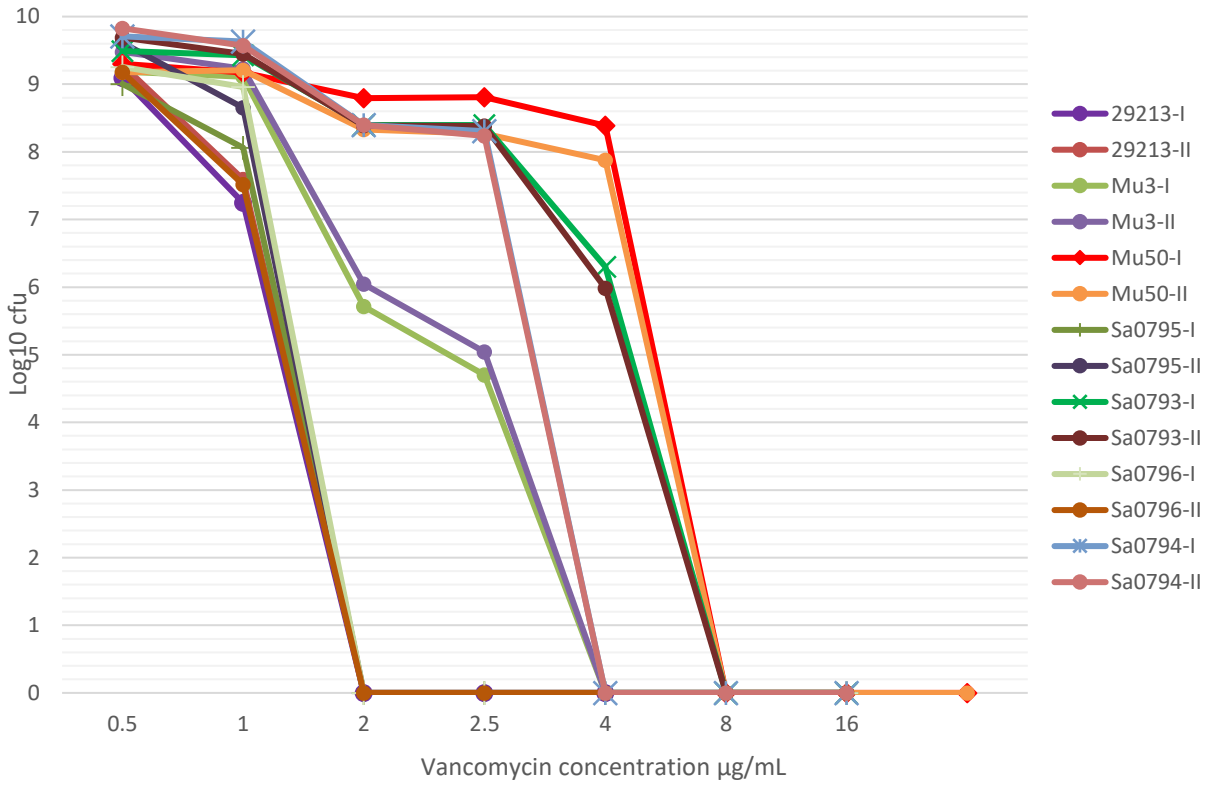


Figure 3.1 Population analysis profile. Log_{10} cfu grown in the presence of vancomycin for the ATCC quality control strains and the known tolerant strains. Isolates tested in duplicate.

Chapter 4

Results

Time-kills

4.1 Introduction

Time-kills are considered a more reliable test for determining tolerance, as the time-kill curve technique determines the actual rate which bacteria are killed, but also has the best correlation with overall cure (CLSI 1999). There are variations in interpreting a time-kill curve, with the CLSI defining tolerance as < 3-$\log_{10}$ reduction in bacterial count at 24 h, while other studies suggest determining the reduction in bacterial count within the first 6-8 h, and using a threshold of line of regression and calculating the kill at 6 h (May, Shannon *et al.* 1998).

4.2 Determining the vancomycin concentration for time kills

Time kills were performed on a representative VSSA (Sa0484 – clinical strain), hVISA (Mu3) and VISA strain (Mu50) and were performed at 1X, 2X, 4X, and 8X the organism's respective MIC, determined by previous MIC tests (Figure 4.1).

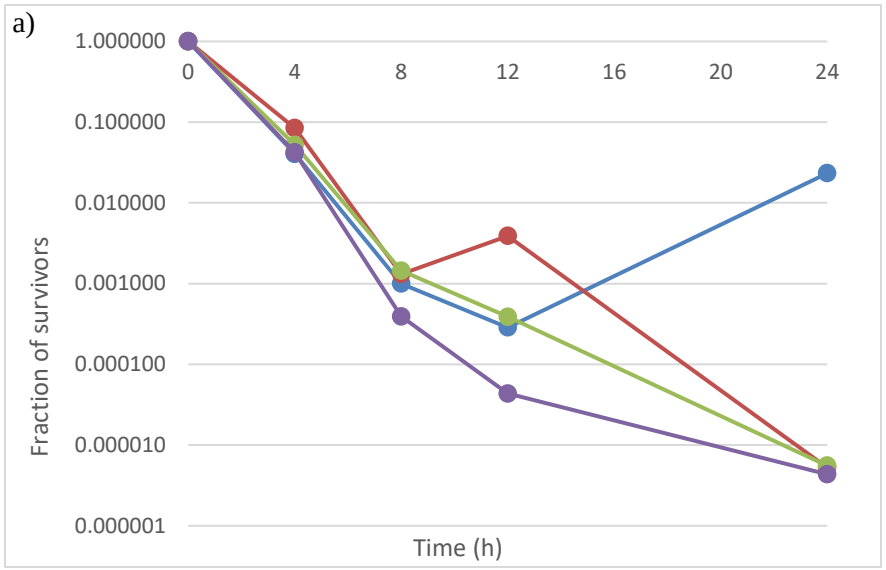
Bacterial regrowth was seen in the VSSA strain Sa0484 at 1X vancomycin MIC (24 h time point) and 2X vancomycin MIC (12 h time point). There was no difference in killing when the concentration was at 4X (8 µg/mL) and 8X (16 µg/mL) vancomycin MIC.

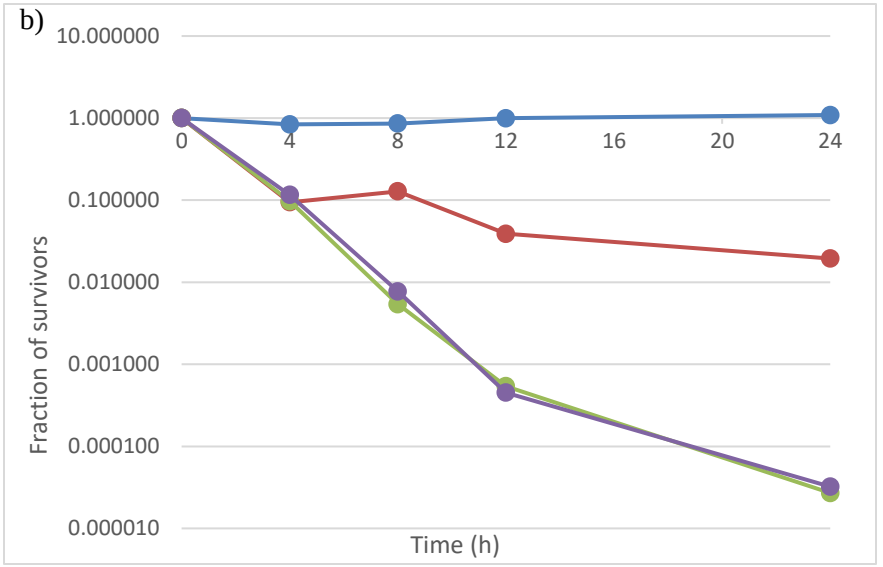
Time kills performed at 1X-2X vancomycin MIC for the hVISA strain Mu3 demonstrated reduced efficacy of vancomycin killing, indicating that the sub-population of VISA strains were resisting the bactericidal activity of vancomycin at these concentrations, and that a minimum vancomycin concentration of $\geq$8 µg/mL is required for killing. There was no difference in killing of Mu3 at vancomycin concentrations of $\geq$ 8 µg/mL.

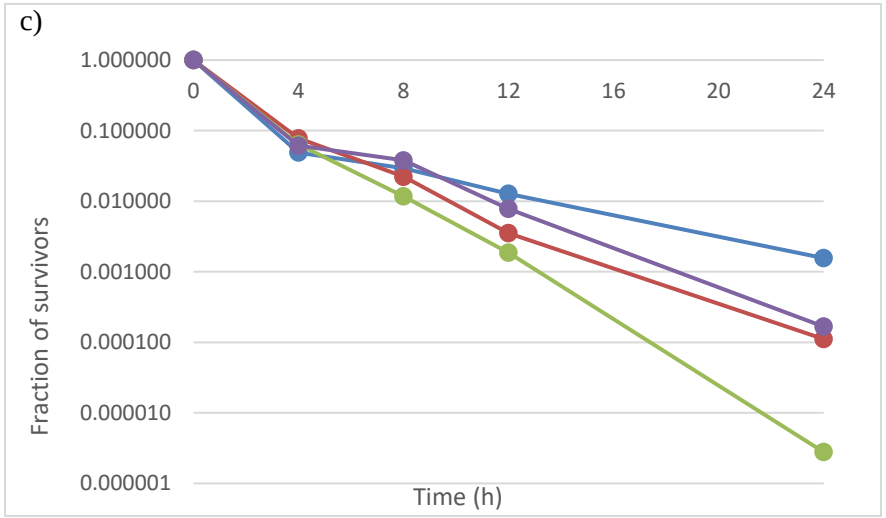
There was no significant difference in killing of the VISA strain Mu50, irrespective of the vancomycin concentration used.

These time-kill results demonstrate that the effect of vancomycin killing is unchanged once the vancomycin concentration is $\geq$ 16 µg/mL, irrespective of whether the strain is a VSSA, hVISA or VISA isolate. As vancomycin concentrations of 16 µg/mL are used in other time kill studies (May, Shannon *et al.* 1998, Sakoulas, Moise-Broder *et al.* 2004, Moise, Sakoulas *et al.* 2007), this therefore was used for further time-kill tests in this study.

Checks for antibiotic carry-over in all vancomycin concentrations used showed no zones of clearing. By allowing 15 minutes for antibiotic absorption into the agar, this indicated antibiotic carry-over was not a problem, even when the vancomycin concentrations were 8X the organism's MIC. Therefore no additional inactivation or wash steps were necessary.







Legend

Concentration	µg/mL vancomycin (a)	(b)	(c)
1X MIC	2	2	8
2X MIC	4	4	16
4X MIC	8	8	32
8X MIC	16	16	64

Figure 4.1 Time-kill data for *S. aureus* strains grown in various vancomycin concentrations. Isolates Sa0484 (VSSA) (a), Mu3 (hVISA) (b), and Mu50 (VISA) (c). Kill rate monitored over times 0, 4, 8, 12, and 24 hours.

4.3 Time-kills

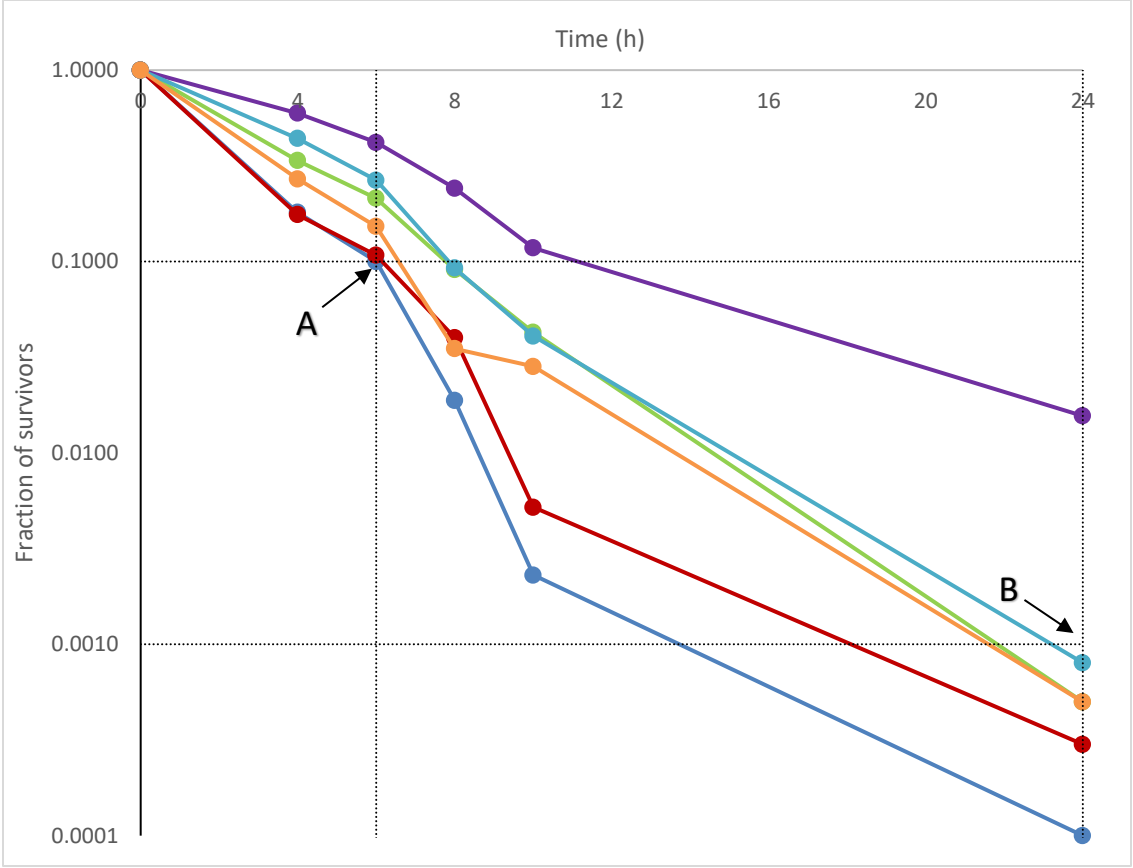
Time-kills were performed on ATCC control strains 29213 (VSSA), Mu3 (hVISA), Mu50 (VISA), tolerant strains Sa0793 (220-post), Sa0794 (225-post) (VISA), and clinical strain Sa0066 (VISA). Time-kills were performed at a fixed vancomycin concentration of 16 µg/mL (Figure 4.2). Checks for antibiotic carry-over were performed and was not detected.

Time kills demonstrated that VISA isolates were killed significantly slower than VSSA and hVISA isolates. Tolerance was only confirmed in a single isolate (Sa066) using the CLSI's definition of tolerance, with 1.56% viable colonies remaining from the starting inoculum at 24 h (Table 4.1). In contrast, using the MDK_{90} of 6 h confirmed that all the reduced vancomycin susceptible isolates were tolerant with the proportion of surviving colonies of Mu3, Mu50, Sa0066, Sa0793 and Sa0794 of 10.77, 21.4, 41.84, 26.6 and 15.24% respectively. The VSSA strain was close to the cut-off for tolerance, with 9.99% viable colonies at 6 h. This data suggests the utility of using the MDK_{90} for defining tolerance, especially given the prevalence of tolerance seen in VISA and hVISA isolates (Jones 2006, Cazares-Dominguez, Cruz-Cordova et al. 2015).

Table 4.1: Time-kill counts for isolates 29213, Mu3, Mu50, Sa0066, Sa0793 and Sa0794.

Time (h)	Percentage of viable colonies (%)					
	29213	**Mu3**	**Mu50**	**Sa0066**	**Sa0793**	**Sa0794**
0	100	100	100	100	100	100
4	18.05	17.59	33.69	59.56	43.90	26.98
6[a]	9.99	10.77	21.40	41.84	26.60	15.24
8	1.88	4.00	9.08	24.13	9.24	3.50
10	0.23	0.52	4.26	11.78	4.08	2.83
24	0.01	0.03	0.05	1.56	0.08	0.05

[a]Count at 6 h was extrapolated using linear regression between times 4 and 8 h.



Legend

	Isolate Number	Phenotype	Tolerance	
			MDK_{90} (A)	CLSI (B)
●	29213	VSSA	NO	NO
●	Mu3	hVISA	YES	NO
●	Mu50	VISA	YES	NO
●	Sa0066	VISA	YES	YES
●	Sa0793	VISA	YES	NO
●	Sa0794	VISA	YES	NO

Figure 4.2 Time-kill data for *S. aureus* strains. VSSA: vancomycin susceptible *S. aureus*; hVISA: heterogeneous vancomycin intermediate *S. aureus*; VISA: vancomycin intermediate *S. aureus*. Kill rate

was monitored over times 0, 4, 8, 10, and 24 hours. Count at 6 h was extrapolated using linear regression between times 4 and 8 h.

An isolate is regarded as tolerant by the CLSI if there was less than 3 log reduction in bacterial population at 24 h following exposure to an antibiotic (depicted by point B); an alternate definition is an MDK_{90} of greater than 6 h, as suggested by May *et. al.* (depicted by point A).

4.4 Time-kills - comparing alternative solid media

CLSI recommend using HBA for MBC and time-kill testing, but some studies use media other than HBA (Table 10.1). To ascertain whether the choice of solid media affects the ability to detect tolerance, Luria broth agar (LBA), an inexpensive plate medium commonly used for the cultivation of bacteria, was used as a comparator against HBA. Time-kills were performed on ATCC control strains 29213 (VSSA), Mu3 (hVISA) and Mu50 (VISA) at a fixed vancomycin concentration of 16 µg/mL (Figure 4.3). Checks for antibiotic carry-over were performed and was not detected, irrespective of the plate media used.

Using the CLSI definition, isolates Mu3 and Mu50 were tolerant when plated on HBA, with 0.22% and 0.13% viable colonies remaining at 24 h (Table 4.2). In contrast, these same strains showed 0.02% viable colonies remaining at 24 h when plated on LBA, classifying them as non-tolerant. Isolate 29213 showed 0.08% and 0.02% remaining colonies at 24 h when plated on HBA and LBA respectively, demonstrating non-tolerance irrespective of the plate media used.

With the exception of a singular 4 h time point for isolate 29213 (HBA: 14.5% viable colonies; LBA: 14.8%), the number of viable colonies were lower for all strains when plated on LBA compared to HBA (Table 4.2). This was marked for Mu50, a known VISA strain, comparing HBA versus LBA at 4 h (60.6% versus 44.25% respectively), at 6 h (50.27% versus 29.37%), 8 h (39.91% versus 14.55%), and 24 h (0.13% versus 0.02%) yielded a large disparity between the number of remaining survivors at each time point.

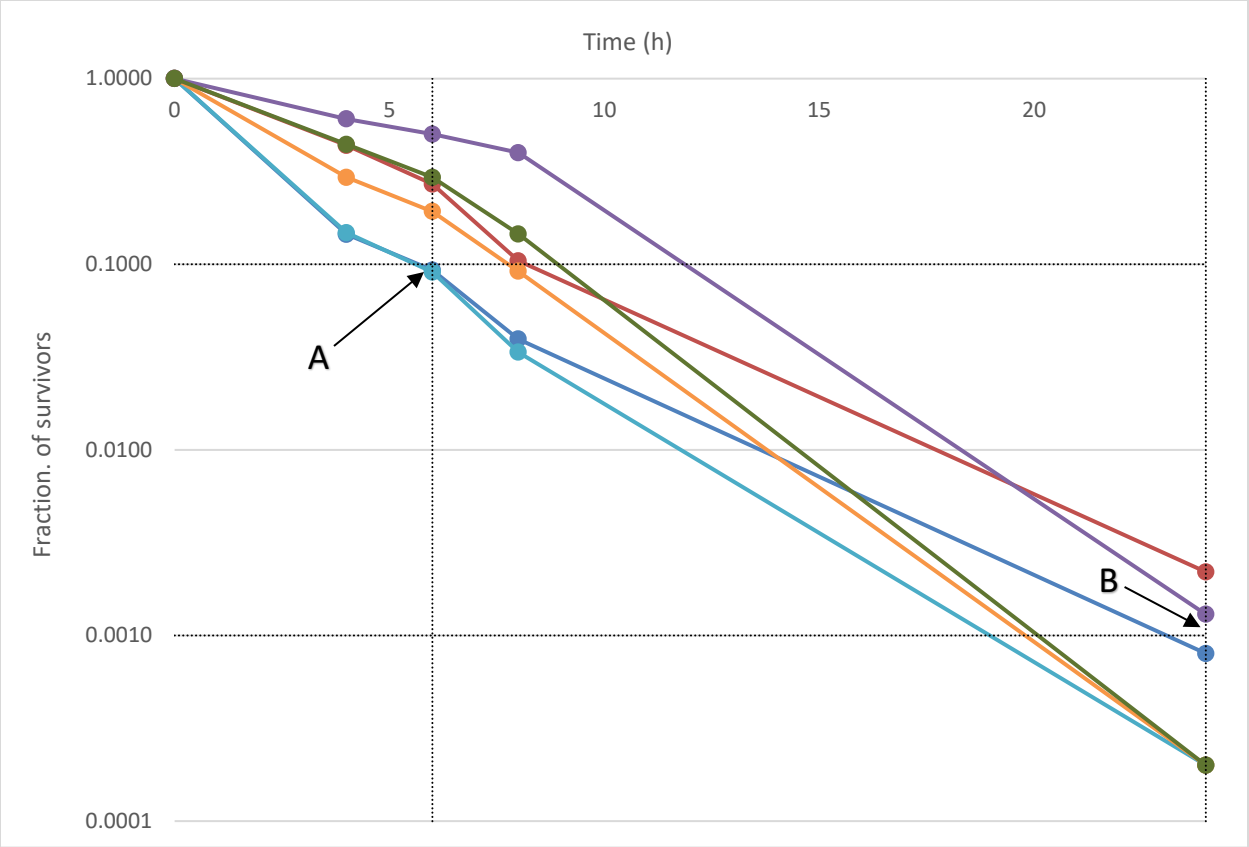
Overall, these lower counts did not impact the classification of tolerance when using the MDK_{90} of 6 h as the criteria for tolerance. Isolates Mu3 and Mu50 were classified as tolerant and isolate 29213 was non-tolerant when using the MDK_{90} of 6 h, irrespective of the plate media used.

Table 4.2: Time-kill counts for isolates plated on HBA and LBA.

	Percentage of viable colonies (%)					
Time (h)	**29213**		**Mu3**		**Mu50**	
	HBA[1]	**LBA[1]**	**HBA[1]**	**LBA[1]**	**HBA[1]**	**LBA[1]**
0	100	100	100	100	100	100
4	14.50	14.80	43.68	29.38	60.62	44.25
6[a]	9.29	9.08	27.05	19.27	50.27	29.37
8	3.95	3.36	10.43	9.17	39.91	14.55
24	0.08	0.02	0.22	0.02	0.13	0.02

[1]Abbreviations: HBA, (horse blood agar); LBA, (Luria broth agar);

[a]Count at 6 h was extrapolated using linear regression between times 4 and 8 h.



Legend

	Isolate Number	Solid media	Phenotype	Tolerance	
				MDK_{90} (A)	CLSI (B)
●	29213	HBA	VSSA	NO	NO
●	Mu3		hVISA	YES	YES
●	Mu50		VISA	YES	YES
●	29213	LBA	VSSA	NO	NO
●	Mu3		hVISA	YES	NO
●	Mu50		VISA	YES	NO

Figure 4.3 Time-kill data for *S. aureus* strains plated on HBA and LBA. VSSA: vancomycin susceptible *S. aureus*; hVISA: heterogeneous vancomycin intermediate *S. aureus*; VISA: vancomycin intermediate *S.*

aureus; LBA: Luria broth agar; HBA: Horse blood agar. Kill rate was monitored over times 0, 4, 8, and 24 hours. Count at 6 h was extrapolated using linear regression between times 4 and 8 h.

An isolate is regarded as tolerant by the CLSI if there was greater than a 3 log reduction in bacterial population at 24 h following exposure to an antibiotic (depicted by point B); an alternate definition is an MDK_{90} of greater than 6 h, as suggested by May *et. al.* (depicted by point A).

Chapter 5

Results

MIC/MBC

5.1 Introduction

MIC testing is well standardised and accepted in clinical laboratories, and the microdilution method was used in this study.

The CLSI recommend an "up to 6 hours for staphylococci" pre-incubation step for MBC testing (CLSI 1999). However greater variability is seen when a 4-5 h pre-incubation step is used, and studies suggest the use of a mid-log phase culture which is typically achieved in 3 h (Taylor, Schoenknecht *et al.* 1983, May, Shannon *et al.* 1998, Harris, Foster *et al.* 2002). For this reason a 3 h pre-enrichment step was used for all of the MIC/MBC tests.

5.2 Tolerance not readily detected when using the CLSI method

In addition to 3 ATCC strains, a total of 63 MRSA strains isolated from patients with sustained bacteraemia seen in Liverpool Hospital were tested for MIC and MBC (Table 5.1). All strains were retrieved from -80ºC storage, and the MICs obtained for all strains replicated MIC results obtained from prior clinical tests, and also matched the respective phenotype which was previously determined by PAP-AUC. The MBCs obtained were largely within 1-2 dilutions of the organism's MIC, and with the exception of Mu50 and Sa0060, tolerance was not seen in these series of isolates. While Mu50 and Sa0060 displayed raised MBC:MIC ratios (16; both isolates), tolerance in these two strains was not readily detected (Mu50: tolerant in 1 of 4 tests: 25% tolerance rate; Sa0060: tolerant in 1 of 2 tests: 50% tolerance rate).

Table 5.1 Tolerance testing CLSI method.

Isolate Number	Phenotype[1]	MIC[1]	MBC[1]	MBC/ MIC[1]	No.[1]	Tolerant % (n)[1]
29213	VSSA	1-2	1-2	1-2	4	0 (0)
Mu3	hVISA	2	4	2	4	0 (0)
Mu50	VISA	8-16	8-128	1-16	4	25 (1)
Sa0012	hVISA	4	4	1	2	0 (0)
Sa0016	hVISA	2	4-8	2-4	2	0 (0)
Sa0018	hVISA	4	8	2	2	0 (0)
Sa0020	hVISA	4	4	1	2	0 (0)
Sa0021	hVISA	4	4	1	2	0 (0)
Sa0022	VSSA	2	4	2	2	0 (0)
Sa0037	hVISA	4	4	1	2	0 (0)
Sa0040	hVISA	4	4	1	2	0 (0)
Sa0044	hVISA	4	4	1	2	0 (0)
Sa0047	VSSA	2	2-4	1-2	2	0 (0)
Sa0048	VSSA	2	2-4	1-2	2	0 (0)
Sa0049	VSSA	2	2	1	2	0 (0)
Sa0050	hVISA	2	4	2	2	0 (0)
Sa0051	hVISA	4	4	1	2	0 (0)
Sa0052	hVISA	2	4	2	2	0 (0)
Sa0053	VSSA	2	2-4	1-2	2	0 (0)
Sa0057	VSSA	2	2-4	1-2	2	0 (0)
Sa0058	hVISA	4	4	1	2	0 (0)
Sa0059	VISA	4	8	2	2	0 (0)

Sa0060	hVISA	8	8-128	16	2	50 (1)
Sa0062	hVISA	1-2	2-4	1-4	2	0 (0)
Sa0063	hVISA	2	4	2	2	0 (0)
Sa0064	hVISA	2	4	2	2	0 (0)
Sa0066	VISA	4	8-16	2-4	2	0 (0)
Sa0067	hVISA	4	4-8	1-2	2	0 (0)
Sa0068	hVISA	4	4	1	2	0 (0)
Sa0069	VSSA	1	2-4	2-4	2	0 (0)
Sa0070	hVISA	2-4	4-8	1-4	2	0 (0)
Sa0091	VSSA	1	2	2	2	0 (0)
Sa0112	VSSA	2	2	1	2	0 (0)
Sa0116	VSSA	2	2	1	2	0 (0)
Sa0129	VSSA	0.5-1	1	1-2	2	0 (0)
Sa0131	VSSA	1	2	2	2	0 (0)
Sa0134	VSSA	1	2	2	2	0 (0)
Sa0138	VSSA	2	2-4	1-2	2	0 (0)
Sa0158	VSSA	1	2	2	2	0 (0)
Sa0160	VSSA	2	2-4	1-2	2	0 (0)
Sa0162	VSSA	1	1	1	2	0 (0)
Sa0164	VSSA	1	2	2	2	0 (0)
Sa0192	VSSA	1	2	2	2	0 (0)
Sa0202	VSSA	1	2-4	2-4	2	0 (0)
Sa0209	VSSA	1	2	2	2	0 (0)
Sa0210	VSSA	1	2	2	2	0 (0)
Sa0211	VSSA	1	2	2	2	0 (0)

Sa0212	VSSA	2	4	2	2	0 (0)
Sa0214	VSSA	2	4	2	2	0 (0)
Sa0228	VSSA	2	4	2	2	0 (0)
Sa0265	VSSA	2	2-4	1-2	2	0 (0)
Sa0283	VSSA	2	2	1	2	0 (0)
Sa0294	VSSA	2	4-8	2-4	2	0 (0)
Sa0308	VSSA	2	2	1	2	0 (0)
Sa0322	VSSA	1	1	1	2	0 (0)
Sa0323	VSSA	1-2	2	1-2	2	0 (0)
Sa0324	VSSA	1	2	2	2	0 (0)
Sa0325	VSSA	2	2	1	2	0 (0)
Sa0329	VSSA	1	2	2	2	0 (0)
Sa0330	VSSA	1	2	2	2	0 (0)
Sa0331	VSSA	2	2	1	2	0 (0)
Sa0332	VSSA	2	2-4	1-2	2	0 (0)
Sa0363	VSSA	2	2	1	2	0 (0)
Sa0375	VISA	4	8	2	4	0 (0)
Sa0378	VISA	4-8	4-8	1	4	0 (0)
Sa0484	VSSA	2	4-8	2-4	2	0 (0)

[1]Abbreviations: VSSA, (vancomycin-susceptible *S. aureus*); VISA, (vancomycin-intermediate *S. aureus*); hVISA, (heterogenous vancomycin-intermediate *S. aureus*); MIC, (minimum inhibitory concentration); MBC, (Minimum bactericidal concentration); No., (Total number of tests performed); % (n), (percentage and number of tests positive)

Tolerance was defined when the MIC:MBC ratio of an isolate was $\geq$32, or $\geq$16 if the vancomycin MIC was 8 μg/mL

5.3 Effect of prolonged storage on tolerance

An additional variable known to affect *S. aureus* tolerance is prolonged storage resulting in false negative results (Mayhall and Apollo 1980). This was seen in the supplied isolates from the US (data not shown). Therefore a series of persistent *S. aureus* strains isolated from sustained bacteraemic patients seen at RPAH (n=23) were prospectively tested for tolerance (Table 5.2). With the exception of Sa0747 exhibiting a MIC in the intermediate range (4µg/mL) in 1 of 2 tests, all strains yielded sensitive MIC results which replicated the MICs obtained from prior clinical testing. MBCs for these persistent strains were all within 1-3 dilutions of the organism's MIC and the highest MBC:MIC ratio obtained among these strains was 8, and was only seen in Sa0751. Despite being tested prior to prolonged storage exposure, tolerance was not detected in any of these persistent isolates.

Table 5.2 Tolerance testing – prospective isolates.

Series	Isolate Number	Isolate Type	MIC[1]	MBC[1]	MBC/MIC[1]	No.[1]	Tolerant % (n)[1]
A	Sa0744	Initial	1	2	2	2	0 (0)
A	Sa0745	Persistent	1	4	4	2	0 (0)
A	Sa0746	Persistent	1-2	1-2	1	2	0 (0)
A	Sa0747	Persistent	1-4	1-4	1-4	2	0 (0)
A	Sa0748	Persistent	1	1-2	1-2	2	0 (0)
A	Sa0749	Persistent	1	2	2	2	0 (0)
A	Sa0750	Persistent	1	1-2	1-2	2	0 (0)
A	Sa0751	Persistent	1	2-8	2-8	2	0 (0)
B	Sa0752	Initial	1	2	2	2	0 (0)
B	Sa0753	Persistent	1-2	2	1-2	2	0 (0)

B	Sa0754	Persistent	1-2	2	1-2	2	0 (0)
B	Sa0755	Persistent	1	1-2	1-2	2	0 (0)
B	Sa0756	Persistent	1-2	2	1-2	2	0 (0)
B	Sa0757	Persistent	1-2	2	1-2	2	0 (0)
C	Sa0758	Initial	1	1-2	1-2	2	0 (0)
C	Sa0759	Persistent	1	2	2	2	0 (0)
C	Sa0760	Persistent	1-2	2-4	2	2	0 (0)
C	Sa0761	Persistent	1	1-2	1-2	2	0 (0)
C	Sa0762	Persistent	1	1-2	1-2	2	0 (0)
C	Sa0763	Persistent	1	1-2	1-2	2	0 (0)
C	Sa0764	Persistent	1	4	4	2	0 (0)
C	Sa0765	Persistent	1	2	2	2	0 (0)
C	Sa0766	Persistent	0.5-1	1-4	2-4	2	0 (0)

[1]Abbreviations: MIC, (minimum inhibitory concentration); MBC, (Minimum bactericidal concentration); No., (Total number of tests performed); % (n), (percentage and number of tests positive)

Tolerance was defined when the MIC:MBC ratio of an isolate was $\geq$32, or $\geq$16 if the vancomycin MIC was 8 µg/mL

5.4 Modification to CLSI method did not increase the detection of tolerance

A study which reported a 70% hVISA and 100% VISA tolerance rate referred to using the CLSI method, but it was noted that the entire well volume (0.1mL) was transferred onto a HBA plate in the final inoculation step (Jones 2006), as opposed to the recommended transfer volume of 0.01mL (CLSI 1999).

When using the CLSI method and transferring 0.01mL from the well contents onto HBA, the tolerance rate in this study for the Liverpool hVISA and VISA isolates did not resemble the tolerance rate described by Jones and colleagues (Table 5.3). Therefore, the same variation used by Jones and colleagues was adopted,

and the final transfer step was altered to 0.1mL (transfer of the entire well contents) onto a HBA plate. A series of predominantly hVISA and VISA strains were selected, with the aim of determining whether the volume used in the final step affects the ability to detect tolerance.

Using a final transfer volume of 0.1mL in the MBC test, the MICs for each strain again matched their respective phenotypes, as the method described by Jones and colleagues does not affect the MIC test. MBCs for this isolate series were similarly within 1-2 dilutions of the organism MIC with the exception Sa0066, which displayed a MBC:MIC ratio of $\geq$32 and was classified tolerant in 1 of 2 tests (50% tolerance rate). All other isolates, which included 7 hVISA and 3 VISA strains were non tolerant. One of the VISA strains which was non-tolerant by this method was Mu50, which in comparison to using a final transfer volume of 0.01mL in earlier testing, demonstrated tolerance in 1 out of 4 tests (25% tolerance rate).

Table 5.3 Tolerance testing Jones *et al.* method.

Isolate Number	Phenotype[1]	MIC[1]	MBC[1]	MBC/MIC [1]	No. [1]	Tolerant % (n) [1]
29213	VSSA	2	2	1	1[#]	0 (0)
Mu3	hVISA	2	4-8	2-4	2	0 (0)
Mu50	VISA	8	16	2	2	0 (0)
Sa0059	VISA	4	8	2	2	0 (0)
Sa0061	hVISA	2-4	4	1-2	2	0 (0)
Sa0062	hVISA	2	2-4	1-2	2	0 (0)
Sa0063	hVISA	2	4	2	2	0 (0)
Sa0064	hVISA	2	4	2	2	0 (0)
Sa0065	hVISA	2	4	2	2	0 (0)
Sa0066	VISA	4	8-≥128	2-≥32	2	50 (1)
Sa0069	VSSA	1	2	1-2	2	0 (0)
Sa0070	hVISA	2-4	4	1-2	2	0 (0)

[1]Abbreviations: VSSA, (vancomycin-susceptible *S. aureus*); VISA, (vancomycin-intermediate *S. aureus*); hVISA, (heterogenous vancomycin-intermediate *S. aureus*); MIC, (minimum inhibitory concentration); MBC, (Minimum bactericidal concentration); No., (Total number of tests performed); % (n), (percentage and number of tests positive)

Tolerance was defined when the MIC:MBC ratio of an isolate was ≥32, or ≥16 if the vancomycin MIC was 8 μg/mL

[#]Single test for strain 29213 excluded as a result of contamination.

5.5 Choice of culture media used prior to testing may affect tolerance detection

MBC testing was performed on reported tolerant strains Sa0793 (220-post) and Sa0794 (225-post) supplied by the United States, which had *in-vitro* vancomycin exposure prior to receipt (Rose, Fallon *et al.* 2012). This study confirmed tolerance in these strains in a time-kill test when using MDK_{90} of 6 h as the criteria for tolerance.

MBC tests were performed in tandem on two sets of isolates from the same parent strain: One performed on isolates grown on Mueller-Hinton agar containing 2 µg/mL vancomycin (MHAV), and another performed on a fresh overnight HBA subculture from the colonies grown on MHAV. These strains were tested using the CLSI method with the recommended 0.01mL transfer volume.

The MICs for Sa0793 and Sa0794 were consistently 2 µg/mL, irrespective of the solid media used prior to testing (Table 5.4). The MICs obtained for Sa0793 and Sa0794 classified them as VSSA strains and did not align with the obtained PAP results which classified the strains as VISA.

MBCs for both Sa0793 and Sa0794 were $\geq$128 µg/mL when tested directly from MHAV and the MBC:MIC ratio for both isolates was $\geq$64. Sa0793 was classified tolerant in 2 of 2 tests (100% tolerance rate) and Sa0794 tolerant in 1 of 2 tests (50% tolerance rate).

Despite being cultured onto HBA from a MHAV plate, MBCs were consistently lower (1-3 dilutions from the organism MIC) when the same strains were tested directly from a fresh HBA subculture plate, and in this tests these strains were consequently classified as non-tolerant.

Table 5.4 Tolerance results for known tolerant strains.

Isolate Number	Pre-test culture media[1]	MIC[1]	MBC[1]	MBC/MIC[1]	No.[1]	Tolerant % (n)[1]
Sa0793	HBA	2	8-16	4-8	2	0 (0)
Sa0793	MHAV	2	≥128	≥64	2	100 (2)
Sa0794	HBA	2	4	2	2	0 (0)
Sa0794	MHAV	2	4-≥128	2-≥64	2	50 (1)

[1]Abbreviations: HBA (horse blood agar); MHAV, (Mueller-Hinton agar containing 2 µg/mL vancomycin); MIC, (minimum inhibitory concentration); MBC, (Minimum bactericidal concentration); No., (Total number of tests performed); % (n), (percentage and number of tests positive)

Tolerance was defined when the MIC:MBC ratio of an isolate was ≥32, or ≥16 if the vancomycin MIC was 8 µg/mL

Chapter 6

Results

Modified method – gradual vancomycin exposure to the organism's MIC

6.1 Introduction

Prior to being used in this study, tolerant strains 220 and 225 were non-tolerant upon retesting when retrieved from storage (data not shown – performed by the University of Wisconsin), and tolerance was detected when these strains were exposed to sub-lethal concentrations of vancomycin. Furthermore in this study, tolerance was detected in these strains when performing MBC testing from colonies grown in vancomycin-containing solid media, and tolerance was not detected when MBC testing was performed on the same colonies obtained from solid media that did not contain vancomycin.

To explore the effect of vancomycin-containing culture media has on tolerance, a selection of 2 VSSA, 6 hVISA and 4 VISA strains were passaged in increasing concentrations of vancomycin until the concentration reached the organism's vancomycin MIC (obtained from previous MIC/MBC results in Chapter 5), followed by MBC testing according to CLSI guidelines. VSSA strains ATCC 29213 and clinical strain Sa0484 acted as a negative tolerant controls. The MICs obtained (in µg/mL) which were used as the final vancomycin concentration were as follows: 29213: 2; Mu3: 2, Mu50: 8, Sa0044: 4, Sa0059: 4, Sa0061: 2, Sa0063: 2, Sa0065: 2, Sa0066: 4, Sa0070: 2, Sa0484: 2, Sa0793: 2.

6.2 Tolerance detected, but elevated MICs also seen

Elevated MICs were seen in VSSA strains 29213 and Sa0484 which both displayed MICs of 4 µg/mL (Table 6.1). The raised MIC indicates intermediate resistance to vancomycin and according to CLSI breakpoints, these strains would be classified as VISA. The hVISA strain Sa0065 also displayed an elevated MIC of 4 µg/mL, which would classify this strain as a VISA.

Tolerance was detected in all VISA strains (100%) as the MBCs obtained for these strains ranged from 64-128 µg/mL; facilitating a MBC:MIC ratio of 16-≥32. 4 out of 6 hVISA strains (66.67%) were tolerant, however Sa0044 and Sa0063 displayed tolerance in 1 of 2 tests. All tolerant hVISA strains had MBCs of ≥128 µg/mL and MBC:MIC ratios of ≥32.

Tolerance was not detected in either VSSA strain, however the MBC for 29213 was raised in comparison to the result obtained without pre-exposure to vancomycin (MBC=16 µg/mL when passaged in 2 µg/mL vancomycin; MBC=2 µg/mL when using CLSI method alone). The MBC:MIC ratio for 29213 was also raised when pre-passaged in vancomycin (MBC:MIC=4), though this did not classify the strain as being tolerant.

With the addition of a vancomycin pre-exposure step to the organism's MIC, results resembled the reported tolerance rates seen in another study (tolerance rate: 70% hVISA; 100% VISA) (Jones 2006).

Table 6.1 Tolerance results for isolates passaged in increasing vancomycin concentrations.

Isolate Number	Phenotype[1]	MIC[1]	MBC[1]	MBC/MIC[1]	No.[1]	Tolerant % (n)[1]
29213	VSSA	4	16	4	2	0 (0)
Mu3	hVISA	2	4	2	2	0 (0)
Mu50	VISA	8	≥128	≥32	2	100 (2)
Sa0044	hVISA	4	8-≥128	2-≥32	2	50 (1)
Sa0059	VISA	4	8-64	2-16	2	0 (0)
Sa0061	hVISA	1	2	2	2	0 (0)
Sa0063	hVISA	2	4--≥128	2-≥32	2	50 (1)
Sa0065	hVISA	4	≥128	≥32	2	100 (2)
Sa0066	VISA	4	≥128	≥32	2	100 (2)
Sa0070	hVISA	4	≥128	≥32	2	100 (2)
Sa0484	VSSA	4	8	2	2	0 (0)
Sa0793	VISA	4	≥128	≥32	2	100 (2)

[1]Abbreviations: VSSA, (vancomycin-susceptible *S. aureus*); VISA, (vancomycin-intermediate *S. aureus*); hVISA, (heterogenous vancomycin-intermediate *S. aureus*); MIC, (minimum inhibitory concentration); MBC, (Minimum bactericidal concentration); No., (Total number of tests performed); % (n), (percentage and number of tests positive)

Tolerance was defined when the MIC:MBC ratio of an isolate was ≥32, or ≥16 if the vancomycin MIC was 8 µg/mL

Chapter 7

Results

Modified method – 0.5x vancomycin MIC

7.1 Introduction

As a result of the increased MICs seen in some VSSA and hVISA strains when pre-exposed to vancomycin to the point of an organism's MIC, a secondary modification was investigated to see whether tolerance is still detected when a lower concentration of vancomycin is used during the pre-testing step, without altering the MIC result. Prior to testing, a selection of strains were incubated in BHI containing vancomycin at half the organisms measured MIC. The vancomycin concentration used (at 0.5X MIC) based on previous MICs obtained (in µg/mL) were as follows: 29213: 1, Mu50: 4, Sa0066: 2, Sa0793: 1. Strains were also pre-enriched in BHI without the added vancomycin prior to testing, which acted as a neutral comparator.

7.2 MIC/MBC data

Exposure to 0.5X vancomycin MIC prior to testing did not affect the organism MIC, as the MICs obtained for all strains matched their respective phenotypes (Table 7.1). The MICs obtained for the isolates pre-exposed to vancomycin were comparable to MICs obtained from isolates that underwent pre-enrichment in BHI without vancomycin. Similar to previous results seen in this study, Sa0793 had an MIC of 2 µg/mL despite being classified as a VISA by PAP. This was also seen in Sa0066, also a VISA strain, which had a MIC of 2 µg/mL.

Strains 29213 and Mu3 were non-tolerant irrespective of the pre-testing culture media used. MBC results across the two methods were also comparable, though tolerance was seen more readily in isolates when pre-exposed to vancomycin. Tolerance was detected in 4/4 (100%) VISA strains when pre-exposed to vancomycin, compared to 3/4 (75%) tolerant VISA strains when pre-enriched in BHI without vancomycin.

Sa0793 was tolerant in 2 of 2 tests (100% tolerance rate) when pre-exposed to vancomycin, compared to tolerance seen in 2 out of 4 tests (50% tolerance rate) when pre-enriched in BHI without vancomycin. Tolerance was seen in 1 of 2 tests for Sa0794 (50% tolerance rate) when pre-exposed to vancomycin, but as a result of a lower MBC:MIC ratio seen in the same strain when pre-exposed in BHI without vancomycin (range: 4-8), this organism was non-tolerant using the latter pre-exposure step.

Table 7.1 Tolerance results for isolates grown with and without vancomycin at 0.5x MIC.

Isolate Number	Phenotype	Exposure to vancomycin at 0.5x MIC					Pre-enrichment in BHI				
		MIC	MBC	MBC / MIC	No.	Tolerant % (n)	MIC	MBC	MBC / MIC	No.	Tolerant % (n)
29213	VSSA	1-2	1-2	1-2	4	0 (0)	1	1-2	1-2	4	0 (0)
Mu3	hVISA	2	4	2	2	0 (0)	2	4	2	2	0 (0)
Mu50	VISA	8	≥128	≥16	4	100 (4)	8	≥128	≥16	4	100 (4)
Sa0066	VISA	2-8	≥128	≥32-≥64	4	100 (4)	2-4	≥128	≥32-≥64	4	100 (4)
Sa0793	VISA	2	≥128	≥64	2[#]	100 (2)	2-4	8-≥128	≥64	4	50 (2)
Sa0794	VISA	4	8-≥128	8-≥32	2	50 (1)	4	4-8	1-2	2	0 (0)

Abbreviations: BHI, (brain heart infusion); VSSA, (vancomycin-susceptible *S. aureus*); VISA, (vancomycin-intermediate *S. aureus*); hVISA, (heterogenous vancomycin-intermediate *S. aureus*); MIC, (minimum inhibitory concentration); MBC, (Minimum bactericidal concentration); No., (Total number of tests performed); % (n), (percentage and number of tests positive)

Tolerance was defined when the MIC:MBC ratio of an isolate was ≥32, or ≥16 if the vancomycin MIC was 8 µg/mL

[#]One test set for strain Sa0793 excluded as a result of contamination.

Chapter 8

Results

Modified method – pre-enrichment step

8.1 Introduction

BHI was an enrichment medium used in this study for all *in vitro* vancomycin pre-exposure steps, and BHI was used by the University of Wisconsin when conducting *in vitro* vancomycin passages in an attempt to revive the tolerance phenotype in strains 220 and 225. Although it was used as a neutral comparator in the vancomycin pre-exposure method described in the Chapter 7, results in Table 7.1 showed an increase in the reported tolerance rate in Mu50 and Sa0066 when using pre-enrichment prior to testing (100%; both isolates), when compared to results seen in Tables 5.1 and 5.3 when tested earlier in this study (16.67% and 25% respectively).

Therefore this study investigated another experimental method where isolates underwent a pre-enrichment step in BHI prior to MIC/MBC testing and were tested alongside the CLSI recommended method. Isolates chosen for this study consisted of 4 hVISA, 5 VISA, and 7 VSSA strains which acted as negative controls.

8.2 MIC/MBC data

Pre-enrichment did not affect the MIC for any isolate: the MIC obtained for all isolates were consistent across both methods and matched the organism's respective phenotype.

Tolerance was not detected in any VSSA and hVISA strain using either method, however tolerance was seen in Mu50, Sa0066, Sa0793 and Sa0794 (all VISA isolates), but there were variations in the number of reported tolerant strains and tolerance rate between both methods (Table 8.1). Mu50 was tolerant only when pre-enrichment was used prior to testing (3 of 10 tests; 30% tolerance rate), and Sa0066 was tolerant in 8 of 8 tests (100% tolerance rate) in the pre-enrichment method, compared to 6 of 8 tests (75% tolerance rate) using the CLSI method. Tolerance was detected in Sa0793 in 1 of 10 tests (10% tolerance rate) in the pre-enrichment method compared to 3 of 10 tests (30% tolerance rate) when using the CLSI method, and tolerance was only detected in Sa0794 in 2 of 10 tests (20% tolerance rate) when using the CLSI method. As the MBC:MIC ratio for Sa0794 was between 1-2 when using the pre-enrichment method, this classified the isolate as non-tolerant.

Table 8.1 Tolerance results for isolates with and without enrichment.

Isolate Number	Phenotype	Pre-enrichment in BHI					CLSI method (No enrichment)				
		MIC	MBC	MBC / MIC	No.	Tolerant % (n)	MIC	MBC	MBC / MIC	No.	Tolerant % (n)
29213	VSSA	1	1-2	1-2	10	0 (0)	1	1-2	1-2	10	0 (0)
Mu3	hVISA	2	2-4	2	10	0 (0)	1-2	4	2-4	10	0 (0)
Mu50	VISA	4-8	4->128	1->32	10	30 (3)	4-8	4-32	1-4	10	0 (0)
Sa0048	VSSA	2	2	1	2	0 (0)	2	2	1	2	0 (0)
Sa0050	hVISA	2	4	2	2	0 (0)	2	4	2	2	0 (0)
Sa0055	VSSA	1	1-2	1-2	2	0 (0)	1	2	2	2	0 (0)
Sa0056	hVISA	2	4	2	2	0 (0)	4	4	1	2	0 (0)

Sa0057	VSSA	1	2	2	2	0 (0)	1	2	2	2	0 (0)
Sa0058	hVISA	2	2	1	2	0 (0)	2	2	1	2	0 (0)
Sa0066	VISA	2-4	≥128	≥32	8	100 (8)	2-4	4-≥128	1-≥32	8	75 (6)
Sa0375	VISA	4	4-16	1-4	2	0 (0)	4	4-32	1-8	2	0 (0)
Sa0484	VSSA	1	1-2	1-2	2	0 (0)	1	2	2	2	0 (0)
Sa0793	VISA	4	4-≥128	1-≥32	10	10 (1)	4	4-≥128	1-≥32	10	30 (3)
Sa0794	VISA	2-4	4	1-2	8	0 (0)	2-4	2-≥128	1-≥32	10	20 (2)
Sa0795	VSSA	1	1-2	1-2	2	0 (0)	2	2	1	2	0 (0)
Sa0796	VSSA	2	2	1	2	0 (0)	1	1	1	2	0 (0)

Abbreviations: BHI, (brain heart infusion); VSSA, (vancomycin-susceptible *S. aureus*); VISA, (vancomycin-intermediate *S. aureus*); hVISA, (heterogenous vancomycin-intermediate *S. aureus*); MIC, (minimum inhibitory concentration); MBC, (Minimum bactericidal concentration); No., (Total number of tests performed); % (n), (percentage and number of tests positive)

Tolerance was defined when the MIC:MBC ratio of an isolate was ≥32, or ≥16 if the vancomycin MIC was 8 μg/mL

Chapter 9

Results

Comparing tolerance results across the four methods used

9.1 Introduction

Tolerance results for isolates 29213 (negative control), Mu3 (hVISA control), Mu50 (VISA control), Sa0066 (a known clinical VISA strain), and Sa0793 and Sa0794 (known tolerant strains; grown in the presence of 2 µg/mL vancomycin) across the four methods used in this study were collated and are represented in Table 9.1. The compared methods included: existing methodology recommended by the CLSI guidelines, vancomycin passage to the organism's MIC, vancomycin at 0.5X the organism's MIC, and pre-enrichment in BHI.

The technical factors described with MBC testing were considered across all four methods: while the inoculum size is recognised as an important factor for MBC testing, isolates grown to logarithmic phase (3 h) were measured against a known MacFarland reference standard prior to inoculation in the microtitre tray. Subsequent bacterial counts were verified the follow day and were within the acceptable range of 1-9 x 10^5 cfu/mL. A 0.01 mL volume was subcultured from clear MIC wells and plated on HBA and antibiotic carryover was not found to be a problem. Though the paradoxical effect has been described in *S. aureus* and MBC testing, the paradoxical effect was not seen in this study, even when strains were pre-exposed to vancomycin prior to testing.

9.2 Tolerance results

The negative quality control strain ATCC 29213 was non-tolerant from a total of 35 MBC tests across 4 variations in testing methodology, and Mu3 was also non-tolerant across all methods from a total of 32 MBC tests, despite being classified as an hVISA strain.

Tolerance was only detected in VISA strains and was readily detected if the isolates were pre-exposed to vancomycin prior to testing, regardless of whether it was achieved via serial vancomycin passage or exposure at half the organism's vancomycin MIC. When pre-exposed to any concentration of vancomycin, the tolerance rate for Mu50, Sa0066 and Sa0793 was 100%, and the tolerance rate for Sa0794 was 50%.

The pre-enrichment method detected a lower tolerance rate when compared to the vancomycin pre-exposure method, but was slightly higher overall when compared to the CLSI method. The tolerance rate using the pre-enrichment method for Mu50 was 50%, Sa0066: 100%, and Sa0793: 21%, compared to the tolerance rates seen in the CLSI method which were 17%, 70% and 25% respectively.

The CLSI method detected tolerance in Sa0794 (tolerance rate: 17%). In comparison, the enrichment method was the only method which failed to detect tolerance in this strain.

Table 9.1 Comparability of tolerance results across four methods.

Isolate Number	Phenotype[1]	CLSI method (HBA)		Vancomycin Passage		0.5x Vancomycin MIC		Enrichment method (BHI)	
		No.	Tolerant % (n) [1]	No.	Tolerant % (n) [1]	No.	Tolerant % (n) [1]	No.	Tolerant % (n) [1]
29213	VSSA	15	0 (0)	2	0 (0)	4	0 (0)	14	0 (0)
Mu3	hVISA	16	0 (0)	2	0 (0)	2	0 (0)	12	0 (0)
Mu50	VISA	6	17 (1)	2	100 (2)	4	100 (4)	14	50 (7)
Sa0066	VISA	10	70 (7)	2	100 (2)	4	100 (4)	12	100 (12)
Sa0793	VISA	12	25 (3)	4*	100 (4*)	2	100 (2)	14	21 (3)
Sa0794	VISA	12	17 (2)	2*	50 (1*)	2	50 (1)	10	0 (0)

Abbreviations: BHI, (brain heart infusion); VSSA, (vancomycin-susceptible *S. aureus*); VISA, (vancomycin-intermediate *S. aureus*); hVISA, (heterogenous vancomycin-intermediate *S. aureus*); MIC, (minimum inhibitory concentration); MBC, (Minimum bactericidal concentration); No., (Total number of tests performed); % (n), (percentage and number of tests positive)

Tolerance was defined when the MIC:MBC ratio of an isolate was $\geq$32, or $\geq$16 if the vancomycin MIC was 8 µg/mL

*Includes results obtained when tested directly from a MHA plate containing 2 µg/mL vancomycin.

Chapter 10

Discussion

10.1 Introduction

Variations in MBC testing has led to uncertainty in the results and has questioned the clinical significance of tolerance. Therefore, this study examined the methodology associated with MBC and tolerance testing.

A high prevalence rate of tolerance has been previously reported in hVISA and VISA strains (Jones 2006, Cazares-Dominguez, Cruz-Cordova et al. 2015), therefore this study examined tolerance testing on an *S. aureus* culture library consisting of a number of hVISA and VISA strains, with the expectation that this study will yield similar tolerance rates.

There are ongoing variations in MBC testing methodology post issuance of CLSI guidelines (Table 10.1), therefore the overall aim of this study was to determine how method variations affect the ability to detect vancomycin tolerance in *S. aureus*.

10.2 PAP of control strains

Population-analysis profiling-area under the curve (PAP-AUC) is not a requirement for tolerance testing, but provides a definitive phenotype profile for *S. aureus* strains, particularly when determining whether a strain is an hVISA. Previous PAP-AUC tests (performed prior to this study on isolates from the ARMEG culture collection library) were useful in identifying hVISA and VISA isolates that were suited for this study, as they are associated with sustained bacteraemia (van Hal, Jensen et al. 2012) and vancomycin tolerance (Jones 2006, Cazares-Dominguez, Cruz-Cordova et al. 2015).

PAP-AUC was also used to determine the phenotype for each ATCC control strain that were used as positive and negative controls for all MBC and time-kill tests. All ATCC strains generated PAP-AUC ratios

which confirmed their respective phenotypes and PAP results aligned with what is seen in the literature (Perazzi, Bello et al. 2011).

PAP-AUC was used to determine the phenotype for reported tolerant strains 220 and 225 and results demonstrated a difference in phenotype depending on whether the strains were subjected to an *in vitro* induced tolerance step via serial vancomycin passage. PAP-AUC classified strains Sa0795 and Sa0796 as VSSA, yet when undergoing vancomycin passage, strains Sa0793 and Sa0794 were classified as VISA. This was further emphasised in the MICs obtained for each strain: MIC for strains Sa0795 and Sa0796 ranged from 1-2 µg/mL, and the vancomycin passaged strains Sa0793 and Sa0794 demonstrated MICs up to 4 µg/mL, classifying them closer to the VISA phenotype. This suggests *in vitro* serial passages in vancomycin may facilitate a change in phenotype, most likely as a result of antibiotic pressure.

10.3 Time-kills

Tolerance results varied among the hVISA and VISA strains depending on the definition used in time-kill assays. The CLSI definition of a <3-$\log_{10}$ reduction of the bacterial count after 24 h classified the VISA isolate Sa0066 as tolerant, but failed to classify Mu3 (hVISA), Mu50 (VISA), and the two reported tolerant strains that underwent vancomycin passage Sa0793 and Sa0794 (which were determined to be VISA by PAP-AUC) as tolerant, showing variability at this time point for detecting tolerance in VISA strains. In contrast, using the MDK_{90} of 6 h classified all hVISA and VISA strains as tolerant, suggesting that this a more robust criteria for categorising tolerance. Mu3 was noted as having survivors close to the cut-off (10.8% colonies at 6 h), though this is not surprising considering that the majority of an hVISA population displays susceptible MICs and only small a sub-population possess intermediate resistance to vancomycin (van Hal and Paterson 2011).

Comparing the time-kill data in quality control strains 29213, Mu3 and Mu50 demonstrated consistently lower viable counts if the time-kill assay was plated on LBA compared to being plated on HBA. When

plated on LBA and using the CLSI definition, Mu3 and Mu50 were classified as non-tolerant. This contrasted the tolerance observed when plated on HBA, suggesting that LBA is not nutritious-rich like HBA, and supports the CLSI recommendation of using HBA as solid media. Surprisingly, the number of remaining colonies on HBA at 24 h for Mu3 exceeded Mu50 (0.22% versus 0.13% respectively). One would expect a higher percentage of remaining colonies in a VISA isolate compared to a hVISA, given the higher MICs associated with VISA strains.

Despite the lower counts observed on LBA, using the MDK_{90} of 6 h definition provided better consistency across both plate media used and classified Mu3 and Mu50 as tolerant, which also favours using MDK_{90} of 6 h for the classification of tolerance in a time-kill assay.

The variation in tolerance classification when using the CLSI definition illustrates why this time point is considered too arbitrary. Using the CLSI definition, Mu3 and Mu50 were classified as non-tolerant in two time-kill assays (Figure 4.2 and 4.3 – LBA) and tolerant in another (Figure 4.3 – HBA), suggesting that the 24 h time point does not consistently detect tolerance in documented tolerant strains. In contrast using the MDK_{90} of 6 h classified every hVISA and VISA strain as tolerant, provided they were plated on HBA. Therefore the results from this study suggest that bactericidal activity is better represented within the first 8 h of a time-kill curve, and strongly suggests that using the criteria of MDK_{90} of 6 h is more suited as the breakpoint for defining tolerance.

Antibiotic streak controls were performed for all strains with each time-kill assay, and whilst antibiotic carry-over has been previously described in *S. aureus*, this was found not to be a problem in this study.

10.4 Tolerance testing: MBC method

Using current CLSI guidelines, MBC testing on a large pool of hVISA and VISA strains in addition to *S. aureus* strains isolated from sustained bacteraemia episodes failed to readily produce tolerant strains: only 2 out of 65 isolates were tolerant, and tolerance was unable to be consistently demonstrated in duplicate testing. These results are surprising considering the reported rate of tolerance seen in hVISA and VISA strains in one large study were 70% for hVISA and 100% for VISA isolates (Jones 2006), and another study classified 41.66% of their tolerant strains as hVISA by PAP-AUC (Cazares-Dominguez, Cruz-Cordova et al. 2015). When adopting the same pipetting method as described by Jones and colleagues, the results from this study again failed to replicate the same tolerance rates, and tolerance results were similar to when 0.01mL of the well contents were transferred: 1 of 11 isolates were tolerant, and tolerance was not consistently demonstrated.

Tolerance is thought to be unstable and may diminish upon prolonged storage (Sabath, Wheeler et al. 1977, Mayhall and Apollo 1980) and could explain the negative results seen in this study when using the CLSI method, as all hVISA/VISA isolates used in this study have been stored at -80ºC for periods >10 years. Storage is also presumed to be a factor for the negative tolerance results seen in the tolerant strains from the US (which required *in vitro* vancomycin passage in order to resuscitate the tolerance phenotype). Ideally isolates would be tested in real time, but as the prevalence of hVISA and VISA strains is low (van Hal, Jensen et al. 2012), it would be unachievable to perform MBC testing in real-time on suspected hVISA isolates (particularly when these strains can only be definitively identified by PAP-AUC; a test which is not routinely performed by clinical laboratories). As VISA and hVISA strains are associated with sustained bacteraemia (van Hal, Jensen et al. 2012), this study carried out prospective MBC testing on a series of *S. aureus* strains obtained from 3 patients with sustained bacteraemia. Prospective MBC testing using the CLSI method did not detect tolerance in these strains, which raises further uncertainty with the sensitivity of MBC testing using existing methodology.

This study initially used a BHI pre-enrichment step as a comparator in the vancomycin pre-exposure method, and noted an increase in the reported tolerance rates when compared to results seen using the CLSI method. It was also noted that the additional pre-enrichment step did not adversely affect any MIC results. An overall comparison of both methods suggests increased sensitivity in tolerance detection when using a pre-enrichment step prior to MBC testing using CLSI guidelines; strains Mu50 and Sa0066 showed marked increases reported tolerance rates and Sa0793 demonstrated comparable rates across both methods. Surprisingly, tolerance was detected in Sa0794 only when using the CLSI method, however this was not readily reproduced, suggesting tolerance is an unstable phenotype even if an enrichment step is used. While the results seen in strain Sa0794 demonstrate variability in the expression of tolerance, the overall results suggest BHI may provide additional nutrients and enhances tolerance detection by supporting an organism's ability to express the tolerance phenotype, particularly if the organism was frozen prior to testing.

Reported tolerant strains 220 and 225 supplied from the US were non-tolerant upon repeat testing after subculture from prolonged storage at -80ºC (performed prior to receipt), and tolerance was detected once the strains were exposed to vancomycin *in vitro* via daily passage (also performed prior to receipt). In this study MBC testing on these isolates demonstrated variability in the expression of tolerance: tolerance was better detected when MBC testing was performed on isolates taken directly from vancomycin-containing agar, and tolerance was not detected when these isolates were subsequently subcultured onto HBA prior to MBC testing.

Tolerance was readily detected in hVISA and VISA isolates (including one of the supplied tolerant strains) when pre-exposed to vancomycin to the point of the organism's MIC prior to MBC testing, however exposure to vancomycin at this concentration elevated the MIC for 3 strains. The MICs obtained for ATCC 29213 and Sa0484 (4 and 8µg/mL respectively) is concerning as both strains would be classified as VISA isolates and furthermore, the MIC for 29213 does not correlate with the MICs established by the ATCC. This suggests vancomycin passage to the organism's MIC may generate a false-positive VISA phenotype in a MIC test in isolates that would normally test as vancomycin-susceptible. In a clinical setting this may

discourage the use of vancomycin as therapy and would instead encourage alternative and or additional therapies for successful treatment. Elevated MICs were not seen if the vancomycin passage was limited to 0.5X the organism's MIC, and yielded tolerance rates similar to when the isolates were pre-exposed to 1X their vancomycin MIC (tolerance was detected in all VISA strains).

The reported tolerant strains that were used for this study required vancomycin passage to revive the tolerance phenotype. Similarly, tolerance has also been previously shown to be gained or lost *in vitro* during repeated subculture in the presence or absence of antibiotic (Voorn, Thompson et al. 1994). Therefore it can be hypothesised that tolerance may emerge during clinical therapy (in this case vancomycin therapy) and may be lost in the clinical laboratory upon subculture of the isolate onto non-selective media, resulting from the absence of vancomycin in the media (May, Shannon et al. 1998). The results in this study have demonstrated that tolerance was lost and gained in strains 220 and 225 and was dependant on pre-testing conditions and culture media used prior to MBC testing. Considering that the vancomycin passage step was performed in BHI (which demonstrated the highest yield in tolerance rates for our hVISA/VISA isolates), pre-exposure to vancomycin in BHI may be the optimal growth condition for an organism to express the tolerance phenotype.

MBC testing failed to detect tolerance in any hVISA strain when using the CLSI or pre-enrichment methods. Considering that the CLSI recommended breakpoint for tolerance in a time-kill failed to consistently demonstrate tolerance in Mu3, this could also explain the negative results seen in MBC testing as the 24 h analysis time-point is also used in MBC testing. Additionally when using the MDK_{90} of 6 h as a criteria for tolerance, Mu3 was close to the tolerance cut-off (10.8-27.5% remaining colonies) which may also explain the negative results seen in the MBC test. Tolerance, however, was readily detected in several hVISA strains if they were pre-exposed to vancomycin prior to MBC testing. This is likely due to a selection for the reduced-susceptible sub-population, which is a direct consequence of pre-exposure in vancomycin during the passage step.

Overall, the results of this study demonstrate that the choice of culture media used prior to MBC testing affects the ability to detect tolerance. As laboratories still use variations in methodology for MBC testing (Table 10.1), this study may explain the variability seen in the number of reported tolerance rates. With respect to MBC testing, a pre-exposure step in vancomycin may be the best approach for detecting tolerance and when pre-exposed to vancomycin, the tolerance rate seen in hVISA and VISA strains closely aligned with the tolerance detected via time-kill assays when the MDK_{90} of 6 h was used as the breakpoint for tolerance. In order to avoid the raised MICs seen as demonstrated in the ATCC 29213 vancomycin susceptible strain, it would be recommended to avoid using vancomycin concentrations at the organism's MIC, and instead favour concentrations at half the measured MIC. Pre-exposure to vancomycin did not induce tolerance in the non-tolerant ATCC 29213 strain and was also not seen in any persistent VSSA strains, which suggests that tolerance is not artificially induced *in vitro* in non-tolerant strains, moreover an organism must intrinsically possess the tolerance phenotype in order to express tolerance.

10.6 Conclusion

The variability of MBC test procedures coupled with the various definitions of tolerance were initially significant factors contributing to the different proportion of strains reported as antibiotic tolerant (Sherris 1986). To overcome this deficiency, the CLSI issued testing guidelines in 1999, which have not been revised since. Despite these recommendations, laboratories testing for tolerance since then, have instituted modifications to the method (Table 10.1). These include the use of different solid media and the amount of volume used to determine the MBC. Though the results from this study have demonstrated overall lower counts in a time-kill when organisms are plated on LBA compared to HBA, the effect of these media changes in these other studies are still largely unknown as the recommended testing method is not included as a comparator. Unlike media modifications, volume changes are known to play a significant role in the detection of tolerance due to antibiotic carry-over, especially when volumes >0.01 mL are used compared to the recommended 0.01 mL (Pearson, Steigbigel et al. 1980). Vancomycin is described as a slowly

bactericidal antibiotic, further complicating the analysis and interpretation of tolerance with this antibiotic (Kollef 2007). Taken together, these differences may explain the large variability (between 4 and 50%) in the prevalence of tolerant isolates detected (Reis, Eisencraft et al. 1995, Honda, Doern et al. 2011, Pasticci, Moretti et al. 2011, Rose, Fallon et al. 2012, Gonzalez, Sevillano et al. 2013). An additional variable known to affect *S. aureus* tolerance is prolonged storage resulting in false negative results (Mayhall and Apollo 1980). Although this was evident in the reported tolerant strains which were provided for this study (isolates 220 and 225), prospective MBC testing using the CLSI guidelines on the three patient series *S. aureus* strains isolated from sustained bacteraemia episodes failed to identify tolerance, despite not having stored these strains at -80ºC prior to testing.

This study confirms the need for ongoing standardization of methodology and definitions of tolerance, and that the classification of tolerance by time kill studies may be the optimal method. Furthermore, it could be argued that the MDK_{90} at 6 h is probably the best cut-off to use especially due to the large variability that exists in bacterial growth, antibiotic concentration and culture media after 6 h (Kaye 1980). This is highlighted when considering the results obtained for the hVISA and VISA strains, which are documented tolerant isolates, and had demonstrated tolerance in MBC tests when pre-exposed to vancomycin. In addition, if MIC/MBC testing is to be performed, an enrichment step supplemented with a sub-lethal concentration of vancomycin is required prior to testing.

Irrespective, there are numerous obstacles before further methodological refinement is undertaken. First and foremost is the establishment of a "gold standard" for tolerance. Unfortunately, the genetic basis for tolerance remains to be determined, however, this study suggests that tolerance may be a surrogate for reduced glycopeptide susceptibility as tolerance could not be confirmed in any VSSA isolate using any methods, even when pre-exposed to vancomycin. Optimal timing of testing should be established as should result thresholds for defining tolerance. While the vancomycin MIC breakpoints have been revised in the last decade, there has been no change in defining tolerance despite reduced vancomycin activity and sustained infections being recognized as a major challenge in the treatment of invasive MRSA infections.

A recent review of tolerance suggested similar standardization and has proposed determining the MDK in a time-kill, to distinguish between tolerance, persistence, and non-tolerance (Brauner, Fridman et al. 2016). These principals were employed in this study and would be recommended for any future tolerance methodology recommendations. However, this concept is subject to variations in methodology and the same inherent flaws.

Without better understanding of clinical and microbiologic features of tolerance and optimization of method development resulting in demonstrated consistency, it will remain a significant challenge to identify the clinical implications of tolerance and establish tolerance testing in diagnostic laboratories to improve antimicrobial selection.

In conclusion, current guidelines and methods used for determining antibiotic tolerance are in urgent need of review. The results from this study strongly recommends using the MDK_{90} of 6 h in a time-kill assay over the CLSI recommendation for the classification of tolerance. The results from this study also demonstrate increased sensitivity in detecting vancomycin tolerance in staphylococci when pre-exposing isolates in vancomycin-containing enrichment media. This pilot study proposes a methodological enhancement to tolerance testing which can provide the framework for a large-scale future study, which would be meaningful in determining the clinical impact of vancomycin tolerance on patient outcomes with invasive MRSA infections.

Table 10.1 Tolerance methodologies implemented post 1999.

Solid media used for colony counts	Volume used to plate on solid media (mL)	Comments	Reference
TSA blood agar	0.02		(Honda, Doern *et al.* 2011)
Horse blood agar	0.01		(May, Shannon *et al.* 1998)
Unknown media	Entire well volume	73.9% hVISA and 100% VISA isolates were found tolerant.	(Jones 2006)
Sheep blood agar	0.025		(Sakoulas, Moise-Broder *et al.* 2004)
Tryptic soy agar	0.02		(Aeschlimann, Hershberger *et al.* 1999)
Muller-hinton agar	0.05		(Pasticci, Moretti *et al.* 2011)
Brain heart infusion agar	Unknown		(Rose, Leonard *et al.* 2008)
Tryptic soy agar	Unknown		(Vidaillac, Leonard *et al.* 2009)
Tryptic soy agar	0.05		(Akins and Rybak 2000)
Tryptic soy agar	0.05	2/86 hVISA isolates were found tolerant	(Rybak, Leonard *et al.* 2008)
Unknown media	0.1	69.3% hVISA, 100% VISA and 14.7% clinical	(Sader, Fritsche *et al.* 2006)

		strains were found tolerant	
Muller-hinton + 5% sheep blood agar	Unknown		(Torrico, Gimenez *et al.* 2010)
Blood agar	0.1	6.1% tolerance rate found in clinical strains	(Traczewski, Katz *et al.* 2009)
Blood agar	0.1		(Wootton, MacGowan *et al.* 2006)

10.7 Future studies

This study has recognised there is a need to update the methodology used for tolerance testing, and there is a need for further standardisation among testing laboratories. Furthermore this study has established the utility of using the MDK_{90} of 6 h in a time-kill as a criteria for tolerance, which has demonstrated to be more robust when compared to existing criteria recommended by the CLSI.

The results from this study also recommend the use of a vancomycin pre-exposure step prior to MBC testing. As tolerance is hypothesised to emerge during clinical therapy, a vancomycin pre-exposure step may resemble what happens to the organism *in vivo* and therefore may facilitate expression of the tolerance phenotype in a laboratory setting.

Using recommendations from this thesis, inter-laboratory testing among institutions with similar hVISA/VISA culture libraries may address the variability seen in the reported rates of tolerance. Only via method standardisation and demonstrated inter-laboratory consistency will we be able to ascertain the prevalence of antibiotic tolerance and better understand its clinical relevance.

10.8 Final remarks

The results from this study contribute to the understanding of antimicrobial resistance for which widespread antibiotic use is a contributing factor. Antibiotic resistance is recognised as a significant global threat which is predicted to attribute to 10 million deaths worldwide by 2050, and will be associated with a global cost of 100 trillion USD (O'Neill 2015). Vancomycin was initially used to treat infections with penicillin-resistant *S. aureus* before less-toxic drugs were introduced (Jones 2006). However due to the increasing prevalence of MRSA bacteraemia vancomycin has become most widely used antimicrobial agent for treatment. However, with the advent of reduced susceptibility and resistance to vancomycin seen in *S. aureus* strains, coupled by increasing reports of treatment failure seen in patients with MRSA bacteraemia, highlights why better understanding of the mechanisms of resistance in these strains is needed. However,

without a standardised approach to tolerance testing and clear established guidelines, this is unlikely to occur.

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